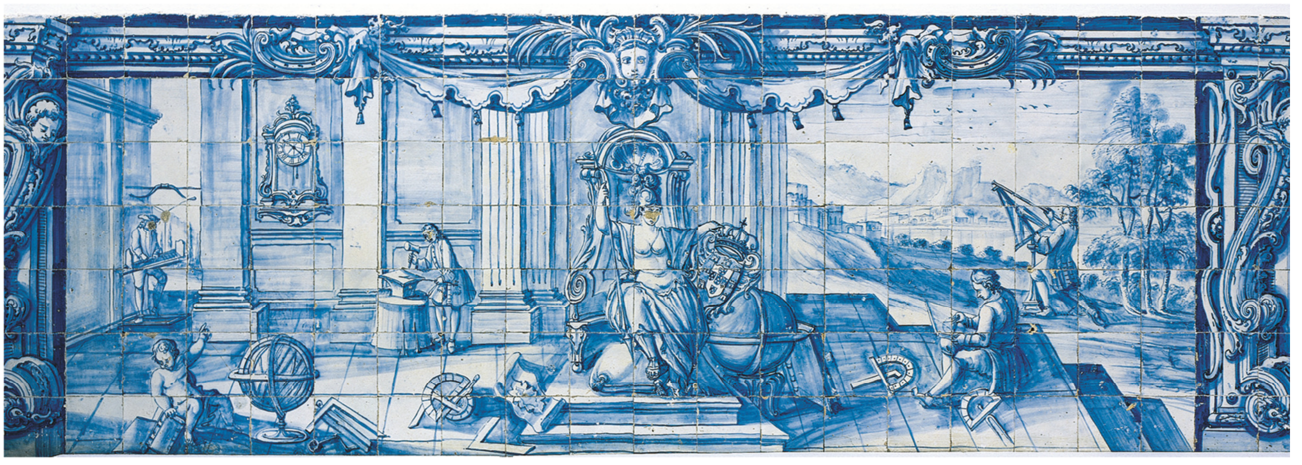
The role of wild leporids as reservoirs of infectious agents

Carina Luísa da Costa Carvalho

Tese apresentada à Universidade de Évora
para obtenção do Grau de Doutor em Ciências Veterinárias

ORIENTADOR (A/ES): *Professora Doutora Elsa Leclerc Duarte*
Doutora Margarida Duarte
Doutora Líbia Zé-Zé

ÉVORA, Outubro de 2017



INSTITUTO DE INVESTIGAÇÃO E FORMAÇÃO AVANÇADA

I dedicate this thesis

To the loving memory of my father Alberto Carvalho

"If I have seen further it is by standing on the shoulders of Giants"

Isaac Newton

Preface

The work presented in this thesis focuses on two important pathogens that affect leporids, the rabbit haemorrhagic disease virus and *Francisella tularensis*. Different aspects of both agents and diseases induced were investigated such as their molecular epidemiology, pathology, laboratorial diagnostics, field monitoring and reservoirs, among others.

The interest in studying Leporids' pathogens was initially motivated by the fact that wild leporids are central species in the Mediterranean ecosystem of the Iberian Peninsula; this is particularly true regarding the European wild rabbit. Secondly, they are also an important source of income as coveted preys in the Portuguese and Spanish significant hunting activity based on small game species. Thirdly, the rabbit meat industries have been expanding their representativeness in Portugal, at least for the past five years, extending the rabbit economic importance, in its domestic type.

With regard to rabbit haemorrhagic disease, the onset of our work auspiciously coincided with the emergence of the new rabbit haemorrhage disease virus 2 (RHDV2) in Portugal. The sanitary importance of the disease induced by this new virus and its complex ecologic and economic effects, quickly drawn our attention towards its study. In nature, RHDV2 has had a tremendous impact in the wild rabbit population and, potentially in the biodiversity of the ecosystems where the species is keystone. Moreover, severe losses have also been recorded in the rabbit industry notwithstanding RHDV2 specific vaccines were developed. In this context, we aimed to contribute to a better understanding of RHDV2 by going a step further in the molecular diagnosis, investigating its genetic variability and evolution through the characterization of strains obtained between 2013 and 2017 in Portugal, as well as achieving a preliminary evaluation of the disease impact in the local rabbit meat industry, with a case-report in a non-vaccinated farm. This research was entirely conducted at the Instituto Nacional de Investigação Agrária e Veterinária (INIAV), virology laboratory, Oeiras, Portugal.

Concerning the zoonotic disease tularaemia, wild hares are considered the main reservoirs and sentinel species for *Francisella tularensis*. Given that hares are important small game species in the Iberia and the concomitant Public Health risk, particularly when disease outbreaks have occurred in the neighbour country of Spain, we intended to verify if this zoonotic bacterium, a potential bioterrorism agent, has been circulating in wild leporids in Portugal. We also investigated which vectors could be more relevant for the disease transmission in our country and attempted a preliminary evaluation of *F. tularensis* epidemiologic reality in Portugal. This research was entirely conducted in Centro de Estudos de Vectores e Doenças Infecciosas (CEVDI), Instituto Nacional de Saúde Doutor Ricardo Jorge (INSARJ), Águas de Moura, Portugal.

This thesis starts with a *General Introduction*, where a global review on the European wild rabbit (*Oryctolagus cuniculus*) and the Iberian hare (*Lepus granatensis*) as well as on the two addressed pathogens, the rabbit haemorrhagic disease virus (RHDV) and *Francisella tularensis*, is presented. A broad-spectrum of topics are revised and discussed in light of the current state of the art allowing the readers to better frame the multiple aspects and implications of the questions addressed. Aspects such as the description of the pathogens and diseases are included in this point. However, the aspects more closely related with the topics investigated in this work are provided by focused and specific reviews of the state of the art that precede each set of studies.

The results obtained during this work were produced to answer specific questions and most are already published in peer-reviewed international journals although some are presently accepted for publication or in preparation. There are eight chapters (I to VIII). Each chapter groups together the studies that fall in the same area of research and starts with a revision of the main aspects related to the topics addressed aiming to facilitate the comprehension of each study. Chapters I to V correspond to research areas related to the rabbit haemorrhagic disease while Chapters VI to VIII refer to research on tularaemia. Altogether, there are 12 studies included in this thesis.

A General Discussion and Future Perspectives follow Chapter VIII and intend to summarize and integrate the main findings of this work, placing the results in a global perspective and providing an overview of the achievements.

References are given altogether in the *References section*, except for the published papers, which are embedding in the text and contain their own reference list.

Finally, the *Annexes* provide additional information on specific topics and publications that were not included in the thesis body, but may be useful information to the reader.

This thesis is based in the following manuscripts and books' Chapters:

Published manuscripts:

Carvalho C.L., Silva S., Gouveia P., Costa M., Duarte E.L., Henriques A.M., Barros S.S., Luís T., Ramos F., Fagulha T., Fevereiro M., Duarte M.D. 2017. Emergence of rabbit haemorrhagic disease virus 2 in the archipelago of Madeira, Portugal (2016-2017). *Virus Genes*.. doi: 10.1007/s11262-017-1483-6. [Epub ahead of print].

Carvalho C.L., Duarte E.L., Monteiro J.M., Afonso C., Pacheco J., Carvalho P., Mendonça P., Botelho A., Albuquerque T., Themudo P., Fevereiro M., Henriques A.M., Barros S.S., Duarte M.D. 2017. Progression of

rabbit haemorrhagic disease virus 2 upon vaccination in an industrial rabbitry: a laboratorial approach. *World rabbit Science* . doi: https://doi.org/10.4995/wrs.2017.5708.

Carvalho C.L., Duarte E.L., Monteiro M., Botelho A., Albuquerque T., Fevereiro M., Henriques A.M., Barros S.S., Duarte M.D. 2017. Challenges in the rabbit haemorrhagic disease 2 (RHDV2) molecular diagnosis of vaccinated rabbits. *Veterinary Microbiology*. 98:43-50. doi: 10.1016/j.vetmic.2016.12.006.

Carvalho C.L., Rodeia J., Branco S., Monteiro M., Duarte E.L., Melo P., Santos P.T., Henriques A.M., Barros, S.S., Ramos F., Fagulha T., Fevereiro M., Duarte M.D. 2016. Tracking the Origin of a Rabbit Haemorrhagic Virus 2 Outbreak in a Wild Rabbit Breeding Centre in Portugal; Epidemiological and Genetic Investigation. *Journal of Emerging Infectious Diseases*. 1: 114. doi:10.4172/24724998.1000114.

Lopes de Carvalho I., Toledo A., **Carvalho C.L.**, Barandika J.F., Respicio-Kingry L.B., Garcia-Amil C., García-Pérez A.L., Olmeda A.S., Zé-Zé L., Petersen J.M., Anda P., Núncio M.S., Escudero R. 2016. *Francisella* species in ticks and animals, Iberian Peninsula. *Ticks Tick Borne Diseases*. 7(1):159-65. doi: 10.1016/j.ttbdis.2015.10.009.

Duarte M.D., **Carvalho C.L.**, Bernardo B., Barros S.V., Benevides S., Flor L., Monteiro M., Marques I., Henriques A.M., Barros S. S., Fagulha T., Ramos F., Luís T., Fevereiro M. 2015. Rabbit haemorrhagic disease virus 2 (RHDV2) outbreak in Azores: disclosure of common genetic markers and phylogenetic segregation within the European strains. *Infection Genetics and Evolution* 35:163-71. doi: 10.1016/j.meegid.2015.08.005.

Duarte M.D., Henriques A.M., Barros S.S., Fagulha T., Ramos F., Luís T., Fevereiro M., **Carvalho C.L.**, Benevides S., Flor L., Barros S.V. and Bernardo S. 2015. Detection of RHDV variant 2 in domestic rabbits in Azores. 2015. *Veterinary Record* 176: 499-500. doi: 10.1136/vr.h2402.

Duarte M.D., **Carvalho C.L.**, Barros S.S., Henriques A.M., Ramos F., Fagulha T., Luís T., Duarte E.L., Fevereiro M. 2015. A real time Taqman RT-PCR for the detection of rabbit hemorrhagic disease virus 2 (RHDV2). *Jornal of Virologic Methods* 219:90-5. doi: 10.1016/j.jviromet.2015.03.017

Carvalho C.L., Zé-Zé L., Lopes de Carvalho I., Duarte E.L. 2014. Tularaemia: a challenging zoonosis. *Comparative Immunology and Infectious Diseases*. 37(2):85-96. doi: 10.1016/j.cimid.2014.01.002.

Manuscripts accepted for publication:

Abade dos Santos F.*, **Carvalho C.L.***, Oliveira N., Correia J.J., Henriques A.M., Peleteiro M.C., Fevereiro M, Duarte, M.D. 2017. Detection of Rabbit Haemorrhagic Disease Virus 2 during the wild rabbit (*Oryctolagus cuniculus*) eradication from the Berlengas archipelago, Portugal. Manuscript accepted for publication in the *BMC Veterinary Research Journal*. *These authors contributed equally to this work.

Manuscripts in preparation:

Carvalho C.L., De Sousa R., Santos-Silva M.M., Santos A.S., Núncio M.S., Lopes de Carvalho I. *Francisella*-like endosymbiont and pathogenic *Ricketsia aeschlimannii* co-infection in *Hyalomma marginatum*. Manuscript in preparation for submission to *Frontiers in Cellular and Infection Microbiology* Journal.

Carvalho C.L., Duarte E.L., Duarte M.D. 2016. Rabbit haemorrhagic virus 2 (RHDV2) was not cultivable *in vitro* after 12 consecutive passages in RK13 and Vero cell lines, similarly to other lagoviruses.

Carvalho C.L., Zé-Zé L., Duarte E.L., Nuncio M.S., Lopes de Carvalho I. 2016. Isolation attempt of *Francisella tularensis holartica* from *Oryctolagus cuniculus* and *Lepus granatensis* tissues after an *in vivo* passage in CD1 mice.

Carvalho C.L., Duarte E.L., Henriques A.M., Barros S.S., Fagulha T., Ramos F., Luís T., Fevereiro M., Duarte M.D. 2017. A review on the geo-phylogeny of RHDV2 (2010-2017).

Book chapters:

Carvalho C.L., Duarte E.L., Zé-Zé L., Lopes de Carvalho I. 2014. Detecção de *Francisella tularensis* pela reacção de polimerase em cadeia em tempo real com sondas de hidrólise TaqMan. In *Abordagens moleculares em veterinária – como desvendar a etiologia e a epidemiologia da infecção*. Edições LIDEL 2014. ISBN: 978-989-752-034-1. pp. 271-276

Lopes de Carvalho I., **Carvalho C.L.**, Núncio M.S. 2012. Tularémia. In *Doenças Associadas a artrópodes vetores e roedores*. Núncio MS & Alves MJ (Eds). Ministério da Saúde. Instituto Nacional de Saúde Doutor Ricardo Jorge, IP. ISBN: 978-972-8643-90-4. pp 99-105.

Online information boletins:

Carvalho C.L., Núncio M.S., Lopes de Carvalho I. 2015. Tularémia – um desafio para Portugal. Online information boletin

Communications:

Carvalho C.L., De Sousa R., Santos-Silva M.M., Santos A.S., Núncio M.S., Lopes de Carvalho I. 2016. Co-infecção de *Francisella*-like endossimbionte e *Rickettsia aeschlimannii* em *Hyalomma marginatum*. II workshop NOVA/INSA de doenças transmitidas por vetores, Lisboa, 19 Oct. Oral communication

Carvalho C.L., De Sousa R., Santos-Silva M.M., Santos A.S., Núncio M.S., Lopes de Carvalho I. 2016. *Francisella*-like endosymbiont and *Rickettsia aeschlimannii* co-infection in a *Hyalomma marginatum* tick. The 20th E-SOVE Conference. Lisbon, Portugal. Oct 3-7. Oral communication

Lopes de Carvalho I., **Carvalho C.L.**, Kingry L.R., Zé-Zé L., Petersen J.M., Núncio M.S. 2014. Detecção de *Francisella tularensis* subsp. *holarctica* em carraças e em lagomorfos. IV Congresso Nacional de Saúde Pública. Lisboa, Portugal. 02-03 Outubro. Oral communication

Lopes de Carvalho I., **Carvalho C.L.**, Kingry L.R., Zé-Zé L., Petersen J.M., Núncio M.S. 2013. Assessment of *Francisella tularensis* in Portugal. International Symposium on *Francisella tularensis* and tularemia. Urgup, Nevsehir, Turkey. July 19-22. Oral communication

Carvalho C.L., Zé-Zé L., Duarte E., Núncio M.S., Lopes de Carvalho I. 2012. Screening of mosquitoes as vectores of *F. tularensis* in Portugal. *VII International conference on Tularemia*. Breckenridge, Colorado, USA, Sept 17-20. Poster

Acknowledgments

During this incredible journey from 2012 to 2017, I have experience the immense pleasure of growing as a research scientist, the amazing satisfaction of small achievements that hard work in Science now-and-then provide, and I faced, and learn to react to, the frustration and disappointment that science work also often offers.

During these 5 years, I was given the privilege of contacting with so many kind, patient, motivated, and knowledgeable people who have helped and encouraged me in the pursuit of my goal that I am afraid to miss any of them. If so, I would like to apologise but I guarantee that in my hearth, no one will be forgotten.

Firstly, I would like to thank to Professor Elsa Leclerc Duarte from the Universidade de Évora (UÉ), for accepting to be my supervisor hence the starting point of this journey. I thank her for the high standard professionalism, for her guidance and scientific support, and for her continued involvement in my work. I am very grateful.

I also would like to thank to Doctor Líbia Zé-Zé from the Centro de Estudos de Vectores e Doenças Infecciosas (CEVDI) for accepting to be my supervisor, for her guidance and scientific support, but also for her kindness, sweetness and for the personal support that she gave me through these years. I am truly thankful for your friendship.

I am also very grateful to Doctor Isabel Lopes de Carvalho from the Centro de Estudos de Vectores e Doenças Infecciosas (CEVDI) for accepting to be my supervisor, for her guidance and expertise and involvement in my work. Thank you very much.

Doctor Margarida Duarte (Instituto Nacional de Investigação Agrária e Veterinária, INIAV), I have been given the opportunity to be your student and I could not ask for a better mentor. I feel that I truly lack the words to thank you and I feel anything that I would say will always fell short. I am truly grateful for your refined professionalism and scientific rigor, for the everyday (and late night!) hard work, for never giving up even when everything seemed difficult or impossible. Thank you for sharing with me the geniality of your scientific mind and your kind hearth. I have learned the world under your guidance.

I would like to thank to Doctor Miguel Fevereiro, Director of the Unidade Estratégica de Investigação e Serviços de Produção e Saúde Animal of Instituto Nacional de Investigação Agrária e Veterinária (INIAV), for accepting me in the Laboratory of Virology, where I could develop the most part of my work. I am truly grateful to all the Laboratory staff who are extraordinary people; Doctors Ana Margarida Henriques and Sílvia Santos Barros, Tiago Luís, Teresa Fagulha and Fernanda Ramos for their help and support during particular moments of my work, technical assistants Maria João Teixeira, Fátima Cordeiro, Arminda da Conceição, Ricardino Ferreira, Rosário Ferreira and Ana Cristina Almeida for their kindness and technical support.

I also thank to Doctor Jacinto Gomes (Laboratory of Parasitology, INIAV)) for his friendship, support and for our interesting discussions during our travels from Setúbal to Lisbon and back home, as well as Doctor Ana Botelho, Dr. Teresa Albuquerque and Dr. Patrícia Themudo (Laboratory of Bacteriology, INIAV)).

I am also very grateful to the Informatics Department of INIAV, namely to Eng.º Valter and to Telmo Poço for their assistance.

I also would like to thank to Doctor Sofia Núncio and Doctor Maria João Alves for accepting me at Centro de Estudos de Vectores e Doenças Infecciosas (CEVDI) where I could develop part of my work. I am very thankful to my sweet and supportive friends Doctor Hugo Osório, Doctor Ana Sofia Santos and Doctor Ana Claúdia Norte. I thank also to Doctors Rita de Sousa, Fátima Amaro and Margarida Santos Silva. I am grateful to all the CEVDI personnel, namely Teresa Luz, Paulo Parreira, Ligia Chaínho, Conceição Paliotes, Ana Maria Mar, Olga Mendes, Paula Melo. My appreciation also to Mr. Luís and Mr. José (Security staff).

I am also thankful to Guilhermina de Jesus Pias for their technical assistance and Doctor Maria João Vila-Viçosa from the Universidade de Évora (UÉ).

I would like to thank to Doctor José Monteiro (Coren) for his collaboration and generosity and to Eng.º António Fernandes (Associação Portuguesa de Cunicultura - ASPOC) for his kindness and sympathy in providing me important data.

I thank to the Divisão de Recurso Cinegéticos e Aquícolas from the Instituto de Concervação da Natureza e Florestas (ICNF).

I would like to thank to Doctors Pedro Melo and Patricia Tavares Santos from Direcção Geral de Veterinária (DGAV). I also would like to thank to Eng.ª Joana Rodeia and Professor Sandra Branco, from the University of Évora.

I also would like to thank to Professor Conceição Peleteiro and her student Fábio Abade dos Santos from the Faculdade de Medicina Veterinária (FMV) as well as to Doctor Nuno Oliveira from Sociedade Portuguesa para o Estudo das Aves (SPEA).

I also would like to thank to Mr. Tavares and his colleagues, namely José Almaça, for receiving me in his hunting station in Alentejo and allowing me to collect samples for my work.

I would like to thank to my good friend Vera Pisco for her support during the hard moments of this journey and for her contagious joy.

I would like to thank to Fundação para a Ciência e Tecnologia (FCT) for my PhD grant (SFRH/BD/79225/2011) and to Instituto de Ciências Agrárias e Ambientais Mediterrânicas (ICAAM) for funding support (UID/AGR/00115/2013).

Finally, I am grateful to my family for all the sacrifices that they've made on my behalf, for exceptional endurance and support during this long and sometimes difficult path. I thank to my mother, Luísa, and my brother Alberto for always supporting me and never let me giving up. I am grateful to my aunts Elvira and São for their part in my education and their love. I thank the world to my husband Pedro and my sweet and dear son Diogo (born in 2013), for enduring this difficult moment of our personal lives. I love you and you make everything being worthwhile. It is in your love that I gather my strengths.

I dedicate this thesis to the loving memory of my kind and sweet father, Alberto. I miss you every day and forever. I know in my hearth that yours intelligent and curious nature would have been absolutely thrilled if we had been giving the opportunity to walk together during this amazing journey of my life.

List of Contents

PREFACE ..... **IX**

ACKNOWLEDGMENTS ..... **XV**

LIST OF FIGURES ..... **XXIX**

LIST OF TABLES ..... **XXXIII**

SUMÁRIO ..... **XLVII**

ABSTRACT ..... **XLIX**

1. GENERAL INTRODUCTION **1**

STRUCTURE OF THE THESIS 3

1.1. THE EUROPEAN WILD RABBIT (*ORYCTOLAGUS CUNICULUS*) 6

1.1.1. Brief evolutionary history *6*

1.1.1.1. Geographic expansion 6

1.1.1.2. Genetic diversity 10

1.1.2. Habitat *13*

1.1.3. Diet *14*

1.1.4. Population dynamics *15*

1.1.5. Rabbit population trends *15*

1.1.5.1. IUCN red list category 17

1.1.5.2. Hunting records to estimate rabbit population abundance 17

1.1.6. The effect of rabbit population dynamics on RHD impact *19*

1.1.7. Importance of the European rabbit in the Iberian Peninsula *19*

1.1.7.1. A keystone species and “engineer” in the Mediterranean ecosystem of the Iberian Peninsula 19

1.1.7.2. An important game species 20

1.1.7.3. An agricultural pest 23

1.1.8. The economic role of rabbit in the industry *23*

1.1.8.1. Relevance of the rabbit meat industry sector in Portugal 24

1.2. THE IBERIAN HARE (*LEPUS GRANATENSIS*) 28

1.2.1 Distribution *28*

1.2.2 Genetic diversity *30*

1.2.3 Morphologic aspects *30*

1.2.4. Habitat and diet *31*

1.2.5. Population dynamics *32*

1.2.6. Importance in the Iberian Peninsula ... *33*
1.2.6.1 Relevance as prey ... 33
1.2.6.2. Relevance as a game species ... 33
1.2.7. Hares as putative sentinels for tularaemia in the Iberian Peninsula ... *33*
1.3. Rabbit haemorrhagic disease (RHD) ... 36
1.3.1. Historic perspective on the emergence of RHDV and RHDV2 ... *36*
1.3.2. Aetiological agent ... *38*
1.3.2.1. Virion structure ... 38
1.3.2.1.1. VP60 (VP1) capsid protein ... 39
1.3.2.1.2. VP10 (VP2) ... 44
1.3.2.1.3. RHDV Core-like particles (CLP) ... 45
1.3.2.2 RHDV genomic organization ... 46
1.3.2.2.1. ORF1 ... 47
1.3.2.2.2. ORF2 ... 48
1.3.2.3. Virus Life Cycle ... 51
1.3.2.3.1. Histo-blood group antigens (HBGAs) attachment factors ... 54
1.3.2.3.1.1. HBGA binding pocket ... 55
1.3.2.3.1.2. Resistance mechanisms to RHDV related to HBGA binding ... 56
1.3.2.3.1.3. Shared human/rabbit ligands for RHDV ... 56
1.3.4. RHD laboratorial diagnosis ... *57*
1.3.5. Information on sanitary status and importation recommendations regarding RHD ... *57*
1.3.6. RHD Pathogenesis and prophylaxis ... *57*
1.3.7. RHD eco-epidemiology ... *57*
1.3.8. RHDV and RHDV2 genetic relations ... *57*
1.3.9. Virus host co-evolution ... *58*
1.4. Tularaemia ... 60
1.4.1. Update on Francisella tularensis phylogeography ... *75*
1.4.1.1 Update on Francisella-like endosymbionts ... *79*
1.4.2. Update on Francisella tularensis epidemiology ... *80*
1.4.3. Update on Francisella tularensis life cycle ... *80*
1.4.4. Update on Francisella tularensis immunopathogenesis ... *80*
1.4.5. Clinical manifestations of tularaemia ... *82*
1.4.5.1. Humans ... 83
1.4.5.2. Animals ... 83
1.4.6. Update on the Francisella tularensis laboratorial diagnosis and genotyping methods ... *84*
1.4.7. Update on tularaemia treatment ... *84*

1.4.8. Update on tularaemia prophylaxis.....85

2. RESULTS OBTAINED: HOW AND IN WHAT WAY HAVE THESE CONTRIBUTED TO OUR KNOWLEDGE OF RHD AND TULARAEMIA **89**

CHAPTER I:.....91

THE RHD LABORATORIAL DIAGNOSIS.....91

1. A review on the RHD laboratorial diagnosis.....*93*

1.1. Sample preparation 93

1.2. Detection Methods 93

1.2.1. Direct diagnostic methods **93**

1.2.1.1. Electron microscopy.....94

1.2.1.2. Antigen detection.....95

1.2.1.2.1. Haemagglutination (HA) test 95

1.2.1.2.2. Monoclonal antibodies (MAb)-based ELISA.....96

1.2.1.2.3 Antigen sandwich ELISA.....97

1.2.1.2.4. Commercial kits.....97

1.2.1.3. Western blotting.....98

1.2.1.4 Molecular methods 98

1.2.1.4.1 Reverse transcriptase polymerase chain reaction (RT-PCR) 98

1.2.1.4.1.1. Conventional PCR 99

1.2.1.4.1.2. Immunocapture-RT-PCR.....99

1.2.1.4.1.3. Real-time RT-PCR.....100

1.2.1.5. Immunostaining.....101

1.2.1.5.1. Immunohistochemical techniques.....102

1.2.1.6. "In-situ" hybridisation (ISH) technique.....102

1.2.1.7. Viral isolation.....102

1.2.2. Indirect diagnostic methods **104**

1.2.2.1. Serologic tests - antibody detection.....104

1.2.2.1.1. Haemagglutination inhibition (HI) test 104

1.2.2.2. Enzyme-linked immunosorbent assay (ELISA) 105

1.2.2.2.1. Indirect ELISA (I-ELISA).....105

1.2.2.2.2. Competition ELISA (C-ELISA) 105

1.2.2.2.3. Isotype enzyme-linked immunosorbent assays (isoELISAs).....107

1.2.2.2.4. Other ELISAs 107

1.2.2.2.5. Serological assays based on specific virus-like particles (VLPs) 108

Study 1.....*109*

Is RHDV2 cultivable and isolable in cell lines?.....*109*

Rabbit haemorrhagic virus 2 (RHDV2) was not cultivable in vitro after 12 consecutive passages in RK13 and VERO cell lines, similarly to other lagoviruses *109*
1. Abstract 111
2. Keywords: 111
3. Body of manuscript 111
4. Acknowledgments 117
5. References 117
Study 2 .... *123*
Can a molecular method for the detection of RHDV2 be developed to assist diagnosis? *123*
A real time Taqman RT-PCR for the detection of rabbit hemorrhagic disease virus variant 2 (RHDV2) *123*
Study 3 .... *131*
What is the meaning of viral charges in the diagnosis of field samples? *131*
Challenges in the rabbit haemorrhagic disease 2 (RHDV2) molecular diagnosis of vaccinated rabbits *131*
CHAPTER II 141
WORLD ORGANISATION FOR ANIMAL HEALTH (OIE) INFORMATION ON RHD SANITARY STATUS AND IMPORTATION RECOMMENDATIONS 141
1. Review on the OIE information on sanitary status and importation recommendations on RHD *143*
1.1. Notification of animal diseases 143
Study 4 .... *145*
What is the importance of early disease report? *145*
Detection of rabbit haemorrhagic disease virus 2 (RHDV2) in domestic rabbits in Azores *145*
CHAPTER III 149
RHD PATHOGENESIS AND PROPHYLAXIS 149
1. Review on RHD pathogenesis *151*
1.1 Classical rabbit haemorrhagic disease virus (RHDV) 151
1.1.1. Adult rabbits 151
1.1.1.1. Clinical symptoms 151
1.1.1.2. Pathological lesions 152
1.1.1.2.1. Macroscopic lesions 152
1.1.1.2.2. Microscopic lesions 153
1.1.1.2.2.1. Liver 153
1.1.1.2.2.2. Lungs and trachea 153
1.1.1.2.2.3. Other organs 153
1.1.1.3. Pathogenesis 154
1.1.2. Young rabbits 155

1.1.2.1. Mechanisms of resistance of young rabbits to RHDV......156
1.1.3. Resistance mechanisms to RHDV......156
1.1.3.1. HBGA polymorphisms......156
1.1.3.1.1. Genes encoding the α1,2-fucosyltransferase implicated in the synthesis of H type 2......157
1.2. Rabbit haemorrhagic disease virus 2 (RHDV2)......158
2. Review on RHD prophylaxis......160
2.1. Active immunization......161
2.1.1. RHDV vaccines......161
2.1.1.1. Inactivated tissue vaccines......161
21.1.1.1 Combined RHDV vaccines......162
2.1.1.2. RHDV subunit recombinant vaccines......162
2.1.1.2.1 Recombinant RHDV-VP60 expressed in baculovirus/Sf9 cell expression system......162
2.1.1.2.2. Escherichia coli recombinant RHDV subunit vaccine......163
2.1.1.2.3. RHDV recombinant vaccines using a myxoma virus vector......163
2.1.1.2.4. Recombinant vaccinia-RHDV virus......164
2.1.1.2.5. Canarypox-based RHDV recombinant vaccine......165
2.1.1.2.6. Insect larvae-derived recombinant RHDV subunit vaccine......165
2.1.1.2.7. RHDV recombinant subunit vaccine obtained from Pichia pastoris......165
2.1.1.2.8. Adenovirus vectored vaccine against RHDV......166
2.1.1.2.9. Expression of the RHDV-VP60 antigen in transgenic plants......166
2.1.2 RHDV2 vaccines......167
2.1.3. Vaccine as post-exposure treatment at the population level......168
2.2. Passive immunization......169
Study 5......171
Is vaccination an important tool for the control of RHDV2 ongoing infections?......171
Progression of rabbit haemorrhagic disease virus 2 upon vaccination in an industrial rabbitry: a laboratorial approach......171
CHAPTER IV......187
RHD ECO-EPIDEMIOLOGY......187
1. Review on RHD eco-epidemiology......189
1.1 Susceptible host species......189
1.2. RHDV survival in the environment......190
1.3. Impact of climate and environmental factors on RHD outbreaks......191
1.4. RHD Transmission......192
1.4.1 The role of insects......193
1.4.2 The role of rabbit-sympatric mammalian species......194
1.4.3. The putative role of vehicles and human movements in the long distance spread of RHDV......194
1.5. The effect of rabbit population dynamics on RHD impact......195

1.6. Management strategies to enhance wild rabbits' populations ... 197
1.6.1 Population monitoring programs ... 198
1.6.2 Hunting pressure ... 198
1.6.3. Predator control ... 199
1.6.4. Habitat management ... 199
1.6.5. Translocations/Restocking and captive breeding in semi-extensive enclosures ... 200
1.6.6. Disease control and vaccination ... 200
Study 6 ... 203
Six years after its emergence, what challenges still poses RHDV2 to the establishment of natural wild rabbit populations? ... 203
Tracking the origin of a rabbit haemorrhagic virus 2 outbreak in a wild rabbit breeding centre in Portugal; epidemiological and genetic investigation ... 203
Study 7 ... 213
Where did the Berlengas - 2016 outbreak virus come from? ... 213
Detection of Rabbit Haemorrhagic Disease Virus 2 during the wild rabbit (Oryctolagus cuniculus) eradication from the Berlengas archipelago, Portugal ... 213
1. Abstract ... 215
2. Keywords: ... 215
3. Introduction ... 215
4. Material and Methods ... 218
4.1 Sample collection ... 218
4.2 Virological examination ... 218
4.3 Nucleotide sequencing analysis ... 219
4.4 Phylogenetic analysis ... 219
4.5 Necropsy and histopathological examination ... 220
5. Results ... 220
5.1. Morphometric data ... 220
5.2 Virological examination ... 220
5.3 Phylogenetic analysis ... 221
5.4 Necropsy and histopathological examination ... 221
6. Discussion and Conclusions ... 222
7. Acknowledgements ... 229
8. References ... 229
CHAPTER V ... 237
RHDV AND RHDV2 GENETIC RELATIONS ... 237
1. Summary on the phylogenetic and evolutionary methods used ... 239
1.1 Phylogenetic analysis ... 239

1.1.1. Models of sequence evolution (DNA substitution) **240**
1.1.2. Methods for investigating the temporal signal and molecular clock of phylogenies **242**
1.1.2.1. The TempEst software 243
1.1.3. Methods estimating phylogenetic trees **243**
1.1.3.1. The neighbor-joining (NJ) method 244
1.1.3.2. Maximum Likelihood 245
1.1.3.3. Bayesian inference of phylogeny 246
1.1.3.3.1. Posterior probabilities (PP) and nonparametric bootstrapping (BS) 247
1.1.4. Other methods used in phylogenetic analysis **248**
1.1.4.1. Networks 248
1.1.4.1.1. Median-Joining networks 249
1.1.4.1.1.1. The Network software 250
1.1.5. The R project for statistical computing **250**
2. RHDV and RHDV2 genetic relations *252*
2.1 Pathogenic lagoviruses 252
2.1.1. RHDV **252**
2.1.1.1 RHDV origin and evolution 252
2.1.1.1.1. RHDV evolution from pre-existing non-pathogenic viruses (NP-LV) 252
2.1.1.1.1.1. Multiple and independent virulent RHDV evolution and emergence 253
2.1.1.1.1.2. Unique emergence of virulence and virulent RHDV evolution 254
2.1.1.1.2. The species jump hypothesis 256
2.1.1.1.3 Recombination in the basis of virulent RHDV emergence 259
2.1.1.2 RHDV genetic diversity 260
2.1.1.2.1 The antigenic variant G6/RHDVa 261
2.1.1.2.2 RHDV phylogeography 264
2.1.1.2.2.1. RHDV genetic diversity in France 264
2.1.1.2.2.2. RHDV genetic diversity in Australia 264
2.1.1.2.2.3. RHDV genetic diversity in the Iberian Peninsula 267
2.1.1.2.2.3.1 RHDV genetic diversity in Portugal mainland 268
2.1.1.2.2.3.2. RHDV genetic diversity in the Azorean archipelago 269
2.1.2. RHDV2 .... **270**
2.1.2.1 RHDV2 origin and evolution 270
2.1.2.1.1. RHDV2 genetic diversity 271
2.1.2.1.1.1. RHDV2 genetic diversity in the Iberian Peninsula 272
2.1.2.1.1.2. RHDV2 genetic diversity in the Azorean archipelago 273
2.1.2.1.1.3. RHDV2 genetic diversity in the Madeira archipelago 275
2.1.2.1.2 RHDV2 phylogeography 275
2.2. Non- and moderately pathogenic lagoviruses (NP-LV) **276**
2.2.1. RCV, Italy 277

2.2.2 Ashigton strain, UK ... 278
2.2.3. Lambay Island strain, Ireland ... 278
2.2.4. Rabbit calicivirus Australia 1 (RCV-A1), Australia ... 278
2.2.5. Michigan rabbit calicivirus (MRCV), US ... 279
2.2.6. The 06-11 strain from France ... 280
Study 8 ... 281
What are the phylogenetic relations between the RHDV2 strains circulating in Azores islands and in Europe? ... 281
Rabbit haemorrhagic disease virus 2 (RHDV2) outbreak in Azores: disclosure of common genetic markers and phylogenetic segregation within the European strains. ... 281
Study 9 ... 293
Are the Madeira archipelago RHDV2 viruses distinct from those characterised so far? ... 293
Emergence of rabbit haemorrhagic disease virus 2 in the archipelago of Madeira, Portugal (2016-2017). ... 293
CHAPTER VI ... 301
TULARAEMIA LABORATORIAL DIAGNOSIS AND GENOTYPING METHODS ... 301
1. Update on the laboratory diagnosis of tularaemia and genotyping methods ... 303
1.1. Culture ... 303
1.2 Serology ... 303
1.3. Molecular methods for diagnosis and genotyping ... 304
1.3.1. Multi-locus sequence typing (MLST) and the gene-by-gene approach ... 304
1.3.2. MLST^{+} based on Whole Genome Sequencing (WGS) ... 305
1.3.3. Genome-wide DNA microarray analysis ... 305
1.3.4. Other molecular methods ... 305
Study 10 ... 313
Is in vivo passage useful in F. tularensis isolation in the presence of low bacteria loads? ... 313
Isolation attempt of Francisella tularensis holarctica from Oryctolagus cuniculus and Lepus granatensis tissues after an in vivo passage in CD1 mice ... 313
1. Abstract ... 315
2. Keywords: ... 315
3. Body of manuscript ... 315
4. Acknowledgments ... 318
5. References ... 318
CHAPTER VII ... 321
EPIDEMIOLOGY OF *FRANCISELLA TULARENSIS* ... 321
1. Update on the epidemiology of Francisella tularensis ... 323
1.1. Mammalian reservoir hosts ... 323

1.2. Arthropod vectors 324

Study 11 ..... *327*

In which mammalian species and vectors do F. tularensis circulates in the Iberian Peninsula? *327*

Francisella species in ticks and animals, Iberian Peninsula *327*

CHAPTER VIII 337

FRANCISELLA-LIKE ENDOSYMBIONTS 337

1. Introduction *339*

1.1. Summary on symbionts classification and transmission patterns 339

1.2. FLEs transmission patterns 340

1.3 Heritable symbiosis 340

1.4. Methods for discriminating between Francisella tularensis and FLEs 341

Study 12 ..... *343*

What role do Francisella-like endosymbionts play in the transmission of vector-borne diseases? *343*

Francisella-like endosymbiont and pathogenic Rickettsia aeschlimannii co-infection in Hyalomma marginatum *343*

1. Abstract 345

2. Body of the manuscript 345

3. Acknowledgements 349

4. References 351

3. GENERAL DISCUSSION 355

4. FUTURE PRESPECTIVES 367

5. REFERENCES 371

6. ANNEXES 417

ANNEX I 419

TULARÉMIA 419

ANNEX II 421

TERRESTRIAL ANIMAL HEALTH CODE (2016) 421

VOLUME II 421

RECOMMENDATIONS APPLICABLE TO OIE LISTED DISEASES AND OTHER DISEASES OF IMPORTANCE TO INTERNATIONAL TRADE 421

CHAPTER 8.17.: TULAREMIA 421

ANNEX III 423

TERRESTRIAL ANIMAL HEALTH CODE (2016) 423

VOLUME II 423

RECOMMENDATIONS APPLICABLE TO OIE LISTED DISEASES AND OTHER DISEASES OF IMPORTANCE TO INTERNATIONAL TRADE 423
CHAPTER 13.2.: RABBIT HAEMORRHAGIC DISEASE 423
ANNEX IV 425
STANDARD METHOD PERFORMANCE REQUIREMENTS (SMPRS) FOR DETECTION OF *F. TULARENSIS* IN AEROSOL COLLECTION DEVICES (AOAC SMPR 2016.007) 425
ANNEX V 427
SCREENING OF MOSQUITOES AS VECTORS OF *FRANCISELLA TULARENSIS* IN PORTUGAL 427
ANNEX VI 429
ACCESSION NUMBERS (GENBANK) OF THE SEQUENCES PRODUCED DURING THIS WORK 429

List of Figures

Figure 1. *Oryctolagus cuniculus* world distribution. This map was based on the IUCN map for the species world distribution and edited according to (Callou, 1995) and (Ferrand and Branco, 2007)...9

Figure 2. (A) and (B) *Oryctolagus cuniculus algirus*, the most abundant subspecies in Portugal (photos taken during wild rabbit translocations in Portugal and kindly relinquished by Doctor Margarida Ramada de Melo)....................10

Figure 3. *O. cuniculus* subspecies distribution in the Iberian Peninsula. The subspecies *O. c. algirus* and *O. c. cuniculus* are essentially allopatric, with a very limited overlap along a north-west/south-east gradient that divides the peninsula (the hybrid zone) (adapted from (Branco et al., 2000)(Alda and Doadrio, 2014))....................12

Figure 4. Mosaic structure of the landscape created by agricultural management (photo taken by Doctor Fílipe Barroso and kindly relinquished by the Instituto de Ciências Agrárias e Ambientais Mediterrânicas (ICAAM))....................14

Figure 5. The distribution of the three hare species that can be found in the Iberian Peninsula. *Lepus granatensis* (gross distribution) is widely distributed in the Iberia (light green colour) while *Lepus castroviejoi* (dark green colour) is restricted to the Cantabrian Mountains and *Lepus europaeus* (medium green colour) occurs along the Pyrenees (adapted from (Campos et al., 2015)). 29

Figure 6. *Lepus granatensis* world distribution. The species is native to the Iberian Peninsula and its geographic range includes Portugal and nearly the entire extent of Spain (yellow colour). *L. granatensis* was introduced in the South of France and Balearic islands (purple colour). This map was based on the IUCN distribution map and edited according to (Acevedo et al., 2012)....................30

Figure 7. The three hare species found in the Iberian Peninsula. (A)– *Lepus granatensis*; (B)- *Lepus castroviejoi*; (C)- *Lepus europaeus* (adapted from (Ballesteros, 1998)). 31

Figure 8. Rabbit haemorrhagic disease virus (RHDV). (A)- Micrograph of purified, negatively stained RHDV (bar=100 nm) (adapted from (Wang et al., 2013); (B)- Reconstructed intact RHDV virion with diameter ~41nm (adapted from (Hu et al., 2010)). 39

Figure 9. Domain organization of RHDV VP60. The Shell (S) domain encompasses amino acids (aa) 66 to 230. The Protruding (P) domain comprehends aa 238 to 579 The P domain is further subdivided in the P1-subdomain, encompassing aa 238 to 287, 450 to 467 and 484 to 579. The P2-subdomain comprehends aa 287 to 450 and 467 to 484 (adapted from (Wang et al., 2013))....................40

Figure 10. Ribbon representation of the crystal structure of the RHDV VP60 S and P domains and topology diagram of P2 subdomain. (A)- S (Shell) domain; secondary structures are coloured blue for helices, gold for β-strands, and grey for loops and are labelled sequentially. (B)- P (Protruding) domain; P1 (green) and P2 (pink) sub-domains are indicated and coloured according to their secondary structure elements (blue for helices, gold for β-strands, and grey for loops) and labelled sequentially. (C)- Topology diagram of the VP60 P2 sub-domain (labels and residue numbers correspond to those shown in panel (B)); (adapted from (Wang et al., 2013))......................................41
Figure 11. RHDV VP60 three-dimensional structures. (A)- Surface-shaded representation of the outer surface of the VP60 capsid viewed along a 3-fold axis of icosahedral symmetry. The positions of the three conformers of VP60 (A, B, and C) are indicated. **(B)**- A model of the VP60 capsid with the front half of the protein shell removed viewed along a 2-fold axis. Six lid-shaped structures located on the inner surface at the 3-fold axis are indicated by arrows. **(C)**- Surface-shaded representation of the outer surface of small NT29 capsids viewed along a 3-fold axis of icosahedral symmetry. T = 1 dimers are shown with the opposite handedness with respect to T = 3 dimers. Two monomers of NT29 protein are indicated (A') (adapted from (Bárcena et al., 2004)). 43
Figure 12. Comparison between intact RHDV virion and CLP. (A)- Central section perpendicular to one of 2-fold axes plotted against the intact RHDV virion and CLP. The RHDV virion contain four regions of densities, from the centre to outside: RNA containing core (O), inner shell (IS), capsid shell (S) and capsid protrusion (P); the arrows indicate the similar features between two structures (adapted from Hu et al., 2010). (B)- Isosurface representation of both intact RHDV virion (diameter ~41nm) and CLP (diameter ~32nm) (adapted from (Hu et al., 2010))...46
Figure 13. Genome organization of RHDV. Both genomic and subgenomic RNA and open reading frames (ORF) 1 and 2 are represented. The translational products and the 257 kDa polyprotein cleavage sites are represented as well (adapted from (Wilblich et al., 1996)(Martín-Alonso et al., 1998) and (Meyers et al., 2000)). 49
Figure 14. Caliciviruses lifecycle. After attachment to the cellular receptor, the virion is internalised into the cell. Uncoating of the viral genome is followed by translation of the polyprotein precursor and co-translational processing releasing the non-structural proteins. These proteins assemble in a replication complex that synthesises the antigenomic RNA, used as a template for the genomic RNA synthesis. The newly synthesized genomic RNA is translated as a polyprotein precursor or is used for packaging in the assembled viral protein core. The antigenomic RNA is also the template for synthesis of subgenomic RNA. The subgenomic RNA is translated as structural proteins, VP60

and VP10. In lagoviruses, VP60 is also released from the polyprotein precursor after processing by the viral protease. Assembly of the structural proteins as well as packaging of the genomic RNA occurs, followed by release of the mature virion from the cell (adapted from (Abrantes et al., 2012)). 53

Figure 15. Phylogeography *Francisella tularensis* subsp. *tularensis* A.I strains from the United States, as obtained by (Birdsell et al., 2014). (A)- Canonical single nucleotide polymorphism (canSNP) of subpopulations. Colours indicate the major subgroups within A.I: A.I.12 is marked in red, A.I.8 in purple and A.I.3 in blue. Subpopulations are indicated by symbols and "n" refers to the number of strains. (B)- Geographic distribution of strains from the subpopulations shown in panel A, by corresponding symbols; top: A.I.12; middle: A.I.8 and bottom: A.I.3. Vertical lines indicate boundaries of the western, central, and eastern regions. Subgroups are mapped based on the geographic origin (state). Darker symbols indicate a higher number of strains; (adapted from (Birdsell et al., 2014))..........76

Figure 16. Geographic distribution of *Francisella tularensis* subsp. *holarctica* groups B.13 (red) and B.FTNF002–00 (purple) (adapted from (Gyuranecz et al., 2012))..........79

Figure 17. RHDV electron microscopy image. This was the first EM photo of RHDV obtained in the Instituto Nacional de Investigação Agrária e Veterinária (INIAV) in 1989. Photo kindly relinquished by Doutora Margarida Duarte (INIAV). 95

Figure 18. Necropsy of RHDV2-positive wild rabbits from Madeira Island (2017). **A**) Gross examination of longitudinal opened trachea showing a haemorrhagic mucosa; **B**) Lung congestion and haemorrhage of the trachea; **C**) Lung (**Lu**) congestion and marbled liver (**Li**). Photos kindly relinquished by Laboratório Regional de Veterinária e Segurança Alimentar (LRVSA), Madeira. 159

Figure 19. Histopathology of a wild rabbit collected during the Madeira Island 2017-RHDV2 outbreak (Haematoxylin & Eosin staining). **A)** Trachea exhibiting congestion of the mucosa (100x). **B)** Lung: congestion of the large vessels (100x). **C)** Liver, hepatocyte karyorrhexis (black arrow) and vacuolar degeneration (white arrow). Greenish brown pigment (hemosiderin) in the cytoplasm of hepatocytes and kupffer cells (200x). **D)** Kidney: picnosis (close-up, white arrows) and karyorrhexis (close- up, black arrow) of tubular cells (400x). Photos kindly relinquished by Laboratório Regional de Veterinária e Segurança Alimentar (LRVSA), Madeira. 160

Figure 20. Fraction of a maximum likelihood (ML) phylogenetic analysis performed by Forrester et al. (2006a) using partial RHDV capsid sequences. Viruses isolated from healthy rabbits are underlined and those isolated from rabbits presumed to have died from RHDV are in normal text.

The "China 84" strain is marked in blue and two of the pathogenic strains from the UK are marked in green ("Ascot" and "Thetford 0199")....................254
Figure 21. Fraction of a maximum likelihood (ML) ML tree of the RHDV capsid gene performed by Kerr et al. (2009), showing the positions of three of the UK isolates sampled before 1984 (written in red) and their close relationship to more contemporaneous isolates....................255
Figure 22. Fraction of the maximum likelihood (ML) tree of the *vp60* capsid gene of lagoviruses performed by Lopes et al. (2014). The RHDV sequences obtained from Iberian hares are marked in red (accession numbers KJ943791 (1996) and KJ943792 (1998)) and group with other RHDV strains from genogroup G1....................258
Figure 23. Neighbor-Joining (NJ) tree performed by Camarda et al. (2014), evidencing that the strain Sr12 RHDV2, the RHDV2 strain infecting the Italian hare (accession number KC741409), clustered together with previous identified RHDV2 strains, forming a distinct clade separated from RHDV and RCV....................259
Figure 24. Phylogenetic tree derived by the neighbor-joining (NJ) method (Le Gall-Reculé et al., 2003) showing that RHDV sequences are clustered into six major genogroups (G1 to G6)....................263
Figure 25. Phylogenetic tree derived by the maximum clade credibility (MCC) method performed by Kovaliski et al. (2014) depicting the evolutionary relationships among the Australian RHDV sequences, forming a monophyletic group with the inoculum strains Czech CAPM V-351....................266
Figure 26. Fraction of the phylogenetic tree derived by the neighbor-joining (NJ) method based on the analysis of the partial vp60 gene from RHDV performed by Müller et al. (2009) depicting that the RHDV strains from Portugal clustered separately from known genogroups and showing the Iberian groups (IB) 1, 2 and 3....................269
Figure 27. Phylogenetic tree by Le Gall-Reculé (2011) using the minimum evolution method derived from partial partial *vp60* sequences of lagoviruses showing that RHDV2 (French RHDV variant) forms a new genetic group, distant from the pathogenic and NP-LV lagoviruses....................272
Figure 28. Fraction of the maximum likelihood (ML) phylogenetic tree of the *vp60* gene of lagoviruses performed by Lopes et al., (2015) showing that the RHDV2 sequences collected in Portugal mainland clustered together with high bootstraps and apart from other lagoviruses, namely RHDV....................273

List of Tables

Table 1. Wild rabbit (*O. cuniculus*) specimens hunted in Portugal mainland between the years 2005 and 2016, during the cinegetic period (from September 1st to December 31st). Data was provided by Dr. Gonçalo Lopes from the Instituto da Conservação da Natureza e das Florestas, IP (ICNF). To compare with the homologous data regarding the Iberian hares (*L. granatensis*) hunted in Portugal during the same period Cf. Table 10, page 35. 18

Table 2. Number of holders of hunting licenses from 2011 to 2016. Data kindly provided by Instituto da Conservação da Natureza e das Florestas, IP (ICNF). 20

Table 3. Number of hunting licenses per type emitted in Portugal from the cinegetic period of 2008/2009 to the homologous period of 2016/2017. Data kindly provided by Instituto da Conservação da Natureza e das Florestas, IP (ICNF). 21

Table 4. Number, type and area in hectars (ha) of the cinegetic zones in Portugal. Data kindly provided by Instituto da Conservação da Natureza e das Florestas, IP (ICNF). 22

Table 5. Data on the industrial meat rabbitry sector in Portugal from 2012 to 2015. The total number of corporations (CAEs) and workers is provided as well as the global turnover (in euros) in the period considered. This information was kindly provided by Eng.º António Fernandes (source: data from Informação empresarial simplificada (IES) - INE - processed by ASPOC). 24

Table 6. Data on the total number of slaughtered meat rabbits in Portugal from 2011 to 2016, by number of animals (x 1000) and carcass weight (t - tonnes). This information was kindly provided by Eng.º António Fernandes (source: data from INE processed by ASPOC). 25

Table 7. Data on the production, importation and exportation of live meat rabbits and on the importation and exportation of rabbit meat from 2011 to 2016 in Portugal. This information was kindly provided by Eng.º António Fernandes (source: data from INE processed by ASPOC) 26

Table 8. Data on the consumption of rabbit meat in Portugal from 2011 to 2016. This information was kindly provided by Eng.º António Fernandes (source: data from INE processed by ASPOC). 27

Table 9. Portugal self-supply rate (rabbit meat), 2013 to 2016. This information was kindly provided by Eng.º António Fernandes (source: data from INE processed by ASPOC). 27

Table 10. Iberian hare (*L. granatensis*) specimens hunted in Portugal mainland between the years 2005 and 2016, during the cinegetic period (from the September 1st to the end of February). Data was provided by Dr. Gonçalo Lopes from the Instituto da Conservação da Natureza e das Florestas,

IP (ICNF). To compare with the homologous data referring to wild rabbits (*O. cuniculus*) hunted in Portugal during the same period Cf Table 1, page 18. 35

List of Acronyms

A	Adenine
aa	amino acid
Acp	Acid phosphatase
A.D.	*Anno Domino* (after Christ)
AF	Adult females
AFLP	Amplified fragment-length polymorphism
AIC	Akaike Information Criterion
AIM2	Absent in melanoma 2 protein
Ala	Alanine
Amplif	Amplification
ANOVA	Analysis of variance
APC	Antigen-presenting cells
AOAC	Aerosol Collection Devices
ASC	Apoptosis-associated speck-like protein
Asp	Aspartate
ASPOC	Associação Portuguesa de Cunicultura
Asn	Asparagine
AST	Antibiotic susceptibility testing
ATG	Initiation codon
AUG	Initiation codon
BamHI	Restriction endonuclease derived from *Bacillus amyloliquefaciens*
BBQ	Quencher molecule
B.C.	Before Christ
BLAST	Basic Local Alignment Search Tool
BHI	Brain-Heart Infusion
BHK-21	Baby hamster kidney cells 21
BIC	Bayesian Information Criterion
BMDM	Bone Marrow-derived Macrophages
bp	Base pair
BS	Bootstrap

BSL-3	Biosafety Level-3
BVFH	BHI supplemented with 2% Vitox, 10% Fildes and 1% Histidine
BTV	Blue tongue virus
BYCE	Buffered charcoal yeast extract
C	Cytosine
CA	Chocolate agar
CAPM-V351	RHDV Czech reference strain
canSNP	Canonical SNP
CEVDI	Centro de Estudos de Vectores e Doenças infecciosas
CDC	Centre for Disease Control and Prevention
cDNA	Complementary deoxyribonucleic acid
CDV	Canine distemper virus
CD4+	Cluster of differentiation 4+
C-ELISA	Competition ELISA
Cf	*Confer*
Cfb	Temperate oceanic climate
CFU	Colony forming unit
CHAB	Cysteine heart agar with blood medium
CI	Confidence interval
CLP	Core-like particles
CO_2	Carbon dioxide
CPE	Cytopathogenic effect
Cq	Quantification cycle
Csa	Hot-summer Mediterranean climate
Csb	Warm-summer Mediterranean climate
cSLAM	Canine signalling lymphocyte activation molecule
Ct	Cycle threshold
CV	Coefficient of variation
cv-farm	Controlled ventilation system farm
CVMP	Committee for Medical Products for Veterinary Use
Da	Number of net nucleotide substitutions per site between populations
Da(JC)	Da with Jukes and Cantor

DAS	Dermacentor andersoni symbiont
DGAV	Direção Geral de Alimentação e Veterinária
DHC	Doença hemorrágica do coelho
DIC	Disseminated intravascular coagulopathy
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded DNA
Dxy	Average number of nucleotide substitutions per site between populations
Dxy(JC)	Dxy with Jukes and Cantor
EBHSV	European Brown Hare Syndrome Virus
ECDC	European Centre for Disease Control and Prevention
E. coli	*Escherichia coli*
ECL	Electrochemiluminescence
EcoRI	Restriction endonuclease enzyme isolated from *Escherichia coli*
EEA1	Early endosomal antigen 1
e.g.	*exempli gratia*
ELISA	Enzyme-linked immunosorbent assay
EM	Electron microscopic
EN	Endangered
ES	Spain
EU-RL	European Union Reference Laboratories
F81 (model)	Felsenstein 1981 (model)
F84 (model)	Felsenstein 1984 (model)
FAM	Carboxyfluorescein (fluorophore or fluorochrome)
F(ab)	Antigen-binding fragment
FBS	Fetal bovine serum
Fcγ	FC (Fragment crystallizable)-gamma receptor
FCP	*Francisella*-containing phagosome
FDA	Food and Drug Administration
FLE	*Francisella*-like endosymbiont
FLE-Am	FLE of *Amblyomma maculatum*
FLA	Free-living amoeba
FNI	*Francisella novicida* Island

*fop*A	*Francisella tularensis* outer membrane protein A gene
FPI	*Francisella* Pathogenicity Island
FR	Fattening rabbits
F. tularensis	*Francisella tularensis*
g	Gravitational force
G	Guanine
G1-G6	Genogroup 1 to Genogroup 6
GBP	Guanylate-binding proteins
GE	Genome equivalents (unit)
GGT	g-glutamil transpeptidase
Glu	Glutamate
Gly	Glycine
gr	grams
gRNA	Genomic ribonucleic acid
GTR (model)	General Time Reversible (model)
GTU	Gene transfer units
HA	Haemagglutination
HAAAP	Hydroxy/aromatic amino acid permeases
HBGA	ABH histo-blood group antigens
H&E	Haematoxylin and eosin
HI	Haemagglutination inhibition
HPD	Highest posterior density
H_2O	Water
H_2O_2	Hydrogen peroxide
HeLa	Immortal cell line derived from cervical cancer from Henrietta Lacks
HRPO	Horseradish peroxidase
IB	Iberian
IBRS-2	Swine kidney epithelial cells
ICAAM	Instituto de Ciências Agrárias e Ambientais Mediterrânicas
ICNF	Instituto da Conservação da Natureza e das Florestas, IP
IC-RT-PCR	Immunocapture reverse transcription-polymerase chain reaction
ICTV	International Committee on Taxonomy of Viruses

IE	Independent estimation
IEM	Immune electron microscopy
IES	Informação Empresarial Simplificada
IgA	Immunoglobulin A
IgG	Immunoglobulin G
Igl	Immunoglobolin lambda
IgM	Immunoglobulin M
iglC	*Francisella tularensis* pathogenicity island protein gene
IL	Interleucine
Ile	Isoleucine amino acid
IFN-α	Interferon-alpha (α)
IFN-γ	Interferon-gamma (γ)
INE	Instituto Nacional de Estatística
INIAV	Instituto Nacional de Investigação Agrária e Veterinária
INSARJ	Instituto Nacional de Saúde Doutor Ricardo Jorge
i.e.	*id est*
I-ELISA	Indirect ELISA
i.d.	Intradermal route
i.m.	Intramuscular route
i.n.	Intranasal route
i.p.	Intraperitoneal route
*ISFtu*2	Insertion sequence-like element (*Francisella tularensis*)
ISH	"*In-situ*" hybridisation
IUCN	International Union for Conservation of Nature
JC (model)	Jukes and Cantor (model)
K	Average number of nucleotide differences
κ	Slope of linear regression
K80 (model)	Kimura 2-parameters (model)
K2P (model)	Kimura 2- parameters (model)
kDa	Kilodalton
Kg	Kilograms
Km	Kilometers

L. castroviejoi	*Lepus castroviejoi*
L. europaeus	*Lepus europaeus*
L. granatensis	*Lepus granatensis*
L. g. gallaecius	*Lepus granatensis gallaecius*
L. g. granatensis	*Lepus granatensis granatensis*
L. g. solisi	*Lepus granatensis solisi*
LAMP	Loop-mediated isothermal amplification
LAMP-1 and 2	Lysosome associated membrane protein 1 and 2
LGS	Library Generation System
LNIV	Laboratório Nacional de Investigação Veterinária
LOD	Limit of detection
Log	Logarithm
*lpn*A	*Francisella tularensis* lipoprotein-coding gene
LPS	Lipopolysaccharide
LRVSA	Laboratório Regional de Veterinária e Segurança Alimentar
LVS	Live vaccine strain
MA-104	Immortal cell line derived from the kidney from African green monkey
Mab	Monoclonal antibody
MAP	Maximum Posterior Probability
MCMC	Markov Chain Monte Carlo
MEM	Minimum Essential Medium
m^7G	7- methylguanosine
$MgCl_2$	Magnesium chloride
mgl	Macrophage growth locus
MHB	Mueller Hinton Broth
MIC	Minimum Inhibition Concentration
MIP	Macrophage inflammatory protein
MIS	Microbial Identification System
MJN	Median Joining Network
ML	Maximum Likelihood
MLVA	Multiple-Locus Variable number tandem repeat Analysis
MLST	Multi-locus sequence typing

mM	Milimolar
MP	Maximum parsimony
MPL	Monophosphoryl lipid
MRCA	Most Recent Common Ancestor
MRCV	Michigan Rabbit Calicivirus
mRNA	Messenger ribonucleic acid (RNA)
MS	Mass spectrometry
msa	Multiple sequence alignment
MSF	Major Facilitator Superfamily
MSN	Minimum Spanning Network
mtDNA	Mitochondrial Deoxyribonucleic acid
MYXV	Myxoma virus
my	Milion years
MyD88	Myeloid differentiation primary response gene (88)
NaCl	Physiological saline solution
NGS	Next Generation Sequencing
NJ	Neighbor-Joining
NK	Natural killer
NNI	Nearest neighbour interchange
Non-Syn	Non-synonimous
NP-LV	Non-pathogenic lagovirus
NS	Non-structural proteins
Ns/s/y	Nucleotide substitutions per site per year
Nt	Nucleotide
NTA	N-terminal arm
Nv-farm	Natural ventilation system farm
NUTS	Nomenclatura das Unidades Territoriais para Fins Estatísticos
O. cuniculus	*Oryctolagus cuniculus*
O. c. algirus	*Oryctolagus cuniculus algirus*
O. c. cuniculus	*Oryctolagus cuniculus cuniculus*
OIE	World Organisation for Animal Health
OPD	o-phenylenediamine

OMP	Outer membrane protein
ORF	Open reading frame
OTU	Operational Taxonomic Units
PBS	Phosphate-buffered saline solution
PCR	Polymerase chain reaction
*pdp*D	*Francisella tularensis* gene encoding a large uncharacterised protein
PFGE	Pulse-field gel electrophoresis
PFMA	Pet Food Manufacturing Association
Pfu	Plaque forming units
pH	Potential of hydrogen
PI	Post-infection
Pi(t)	Nucleotide diversity
PK-15	Pig kidney 15 cell line
PMN	Polymorphonuclear cells
POT	Proton-dependent oligopeptide transporters
PP	Posterior probabilities
PPV-NK	Plum pox virus based vector
PT	Portugal
PTA	Phosphotungstic acid
pur	Purine
PVDF	Polyvinylidene flouride
pyr	Pyrimidine
QSY-7	Quencher molecule
R^2	Coefficient of determination (linear regression)
RBC	Red blood cells
RCV	Rabbit calicivirus
RCV-A1	Rabbit calicivirus Australia 1
RD	Region of difference
RD1	Region of difference 1
rDNA	Ribosomal DNA
RdRp	RNA-dependent RNA-polymerase
REVIVE	Rede Nacional de Vigilância de Vectores

RFLP	Restriction fragment length polymorphism
RHD	Rabbit haemorrhagic disease
RHDV	Rabbit haemorrhagic disease virus
RHDVa	Rabbit haemorrhagic disease virus antigenic variant G6
RHDV2	Rabbit haemorrhagic disease virus 2
RHDVb	Rabbit haemorrhagic disease virus b
RLB	Reverse line blotting
RK-13	Rabbit kidney 13 cell line
Rm	Recombination events
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
ROS	Superoxide anion generation
RT-PCR	Reverse transcriptase reaction followed by polymerase chain reaction
RT-qPCR	Reverse transcriptase reaction-quantitative polymerase chain reaction
Ru(bpy)3(2+)	Cation of the Tris (bipyridine) ruthenium (II) chloride
S.	Saint (São in Portuguese)
SARS	Severe acute respiratory syndrome
s.c.	Subcutaneous route
SD	Standard deviation
*sdh*A	*Francisella tularensis* succinate dehydrogenase gene
SDS	Sodium dodecyl sulphate
SE	Simultaneous estimation
Seq	Sequencing
Ser	Serine
Sf9	*Spodoptera frugiperda* 9
SFG	Spotted fever group
sgRNA	Subgenomic Ribonucleic acid
SIRS	Systemic inflammatory response syndrome
SMPRs	Standard Method Performance Requirements
SMSV	San Miguel sea lion virus
SNP	Single nucleotide polymorphism
SPF	Specific pathogen free

SP-ELISA	Solid-phase ELISA
Splenocyte-BMDM	Splenocyte-Bone Marrow-Derived Macrophages
SR-A	Scavenger receptor A
St	Saint (Santa in Portuguese)
ST1-ST5	Sequence type 1 to Sequence Type 5
STR	Short-tandem repeats
subsp.	Subspecies
Syn	Synonymous
T	Thymine
TCP	Trypsin-like cysteine protease
TGEV	Transmissible gastroenteritis swine coronavirus
Thr	Threonine amino acid
TLR	Toll-like receptor
TMRCA	Time to Most Recent Common Ancestor
TN93 (model)	Tamura-Nei 93 (model)
TNF-α	Tumoral necrosis factor α
T6SS	Type VI secretion system
*tul*4	*Francisella tularensis* lipoprotein encoding gene
TURBS	Termination Upstream Ribosomal Binding Site
Tyr	Tyrosine
U	Uracil
UK	United Kingdom
UNESCO	United Nations Educational, Scientific and Cultural Organization
µl	Microliter
µM	Micromolar
USA	United States of America
UTR	Untranslated region
Val	Valine
VERO	Cell line derived from African green monkey kidney epithelium cells
VESV	Vesicular exanthema of swine virus
VLP	Virus-like particles
VNTR	Variable Number Tandem Repeat

VP60 or VP1	Capsid protein of Rabbit haemorrhagic disease virus
VP10 or VP2	Minor structural protein of Rabbit haemorrhagic disease virus
vp60	Capsid protein gene
VPg	Viral genome-linked protein
vs	*versus*
VT	Verocytoxin
VTEC	*Escherichia coli* strains that produce verocytoxin
WGS	Whole genome sequencing
Π	Nucleotide diversity
2DE	Two-dimensional gel electrophoresis

Sumário

Título da Tese: O papel dos leporídeos silvestres como reservatórios de agentes infeciosos

Os leporídeos silvestres coelho-bravo (*Oryctolagus cuniculus*) e lebre ibérica (*Lepus granatensis*), são determinantes no equilíbrio de vários ecossistemas na Península Ibérica e, simultaneamente, espécies cinegéticas de relevante importância económica.

Esta tese visou investigar o vírus da doença hemorrágica dos coelhos de tipo 2 (RHDV2), detetado em Portugal desde 2012 e atualmente disseminado no continente e arquipélagos (Açores, Madeira, Berlengas). Dado o impacto alarmante nas populações de coelho-bravo, o Governo Português ativou recentemente um plano para controlo da doença (Despacho 4757/2017 de 31 de Maio).

Um segundo objetivo desta tese consistiu na epidemiovigilância da *Francisella tularensis*, uma bactéria patogénica zoonótica transmitida por vetores, com potencial impacto em Saúde Pública. A lebre ibérica é seu reservatório e potencial espécie sentinela.

Os estudos apresentados nesta tese envolveram a utilização de metodologias clássicas de patologia, microbiologia e moleculares. Para a análise de dados produzidos recorremos, entre outros, a inferências filogenéticas, e a programas de análise estatística e bioformática (eg R software).

Este trabalho permitiu o desenvolvimento e validação do primeiro método molecular de diagnóstico para RHDV2, presentemente adotado pelo manual da OIE, e contribuiu para uma compreensão da dinâmica de evolução do vírus em cenários epidemiológicos e geográficos distintos, alargando o conhecimento das relações filogenéticas entre as estirpes que circularam em Portugal entre 2012 e 2017.

No que diz respeito à tularémia, este estudo permitiu aferir a situação epidemiológica da doença em Portugal, confirmando o papel dos leporideos silvestres como reservatório e possibilitando conclusões preliminares sobre o risco desta zoonose para a Saúde pública no país. Adicionalmente, permitiu confirmar que os ixodídeos são o principal vetor de *F. tularensis* em Portugal.

Globalmente, este trabalho contribuiu para o estado-da-arte das duas infeções, e disponibilizou informação relevante para adequar o diagnóstico e a profilaxia sanitária e médica destas duas doenças à realidade atual.

Abstract

Thesis title: The role of wild leporids as reservoirs of infectious agents

The European wild rabbit (*Oryctolagus cuniculus*) and the Iberian hare (*Lepus granatensis*), are keystones species in various ecosystems of the Iberian Peninsula and on the local game-based economy.

This thesis aimed to investigate the rabbit haemorrhagic disease virus 2 (RHDV2), detected in Portugal in 2012 and currently disseminated in the continent and autonomous regions (Azores, Madeira, Berlengas). Given its alarming impact in the wild rabbit populations, the Portuguese Government has recently activated a plan aiming the control of the disease (Despatch 4757/2017 of May 31st).

A second objective of this thesis was the epidemiological surveillance of *Francisella tularensis* zoonotic vector-borne pathogenic bacterium, with potential impact on Human Health. The Iberian hare is considered reservoir and potential sentinel species for this pathogen.

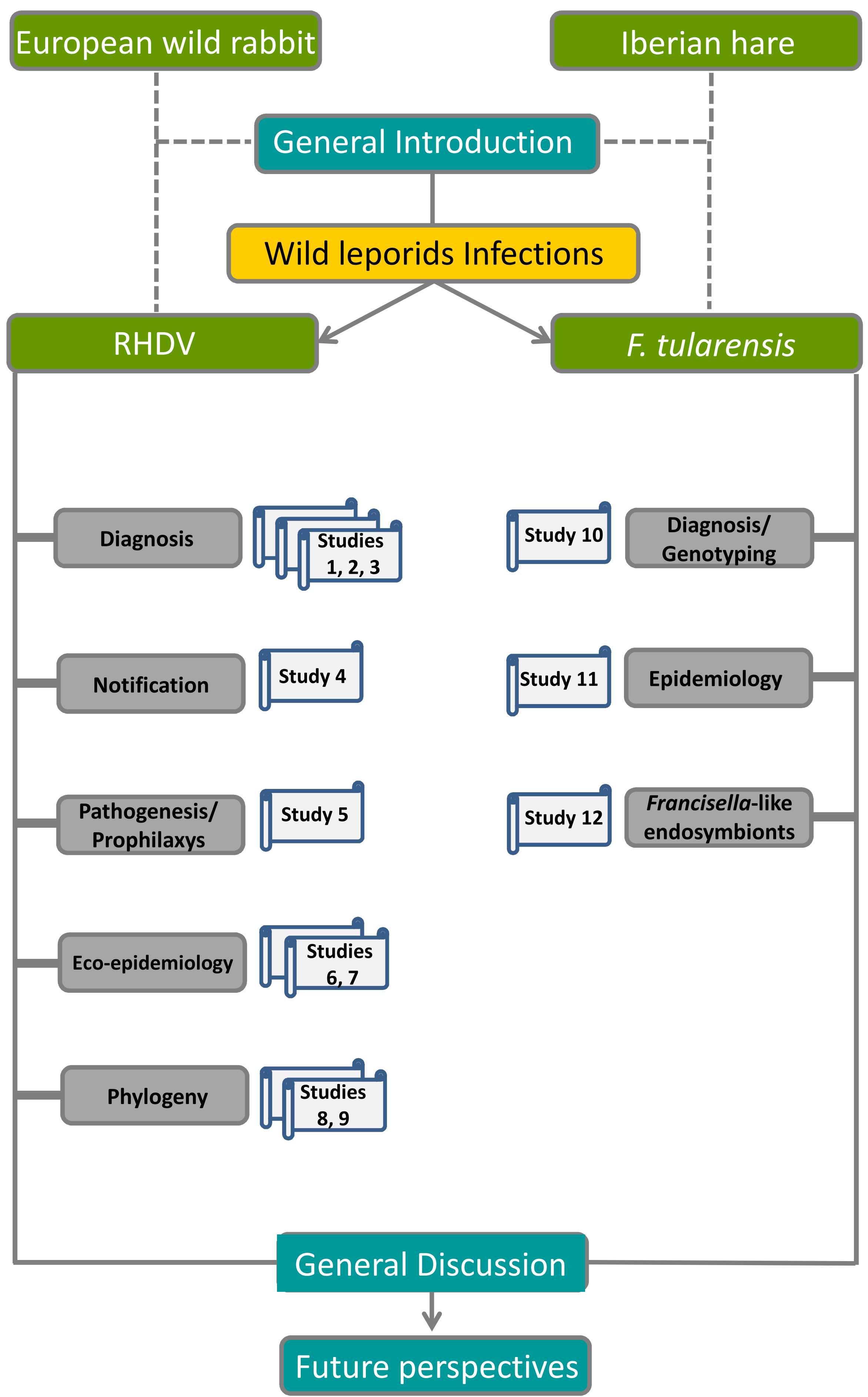
Several methodologies were used to carry out the studies presented in this work, including basic pathology, microbiology and molecular methods. Data analysis involved the resource to phylogenetic inference, statistical programs and bioformatics (e.g. R software).

This work enabled the development and validation of the first molecular diagnostic method for RHDV2, currently figuring in the OIE manual, and allowed insights into to the virus dynamic evolution in different epidemiologic and geographic contexts, widening the comprehension of RHDV2 phylogenetic relations among the strains that circulate in Portugal from 2012 until 2017.

This study also made possible to ascertain the tularaemia epidemiologic situation in Portugal, confirming the role of wild leporids as reservoirs for the agent and enabling preliminary conclusions on Public Health risk in the country. Moreover, this work allowed confirming ticks as the main vectors for *Francisella tularensis* in Portugal.

Globally, this work contributed to the state-of-the-art of both infections and produced relevant information that can be used to adjust the medical and sanitary prophylactic measures of both diseases to the present reality.

1. General Introduction



Structure of the thesis
European wild rabbit
Iberian hare
General Introduction
Wild leporids Infections
RHDV
F. tularensis
Diagnosis
Studies 1, 2, 3
Notification
Study 4
Pathogenesis/ Prophilaxys
Study 5
Eco-epidemiology
Studies 6, 7
Phylogeny
Studies 8, 9
Study 10
Diagnosis/ Genotyping
Study 11
Epidemiology
Study 12
Francisella-like endosymbionts
General Discussion
Future perspectives

The *General Introduction* starts by reviewing the main aspects of the two most central leporids found in Portugal and the Iberian Peninsula, the European wild rabbit (*Oryctolagus cuniculus*) and the Iberian hare (*Lepus granatensis*). The European wild rabbit evolutionary history, habitat, diet and population dynamics as well as the species relevance in the Iberia, particularly as key species and "engineer" of the Mediterranean ecosystem and coveted prey in small game hunting, are addressed. Also, key aspects on the Iberian hare including the species distribution and genetic diversity, habitat and diet, population dynamics, relevance as prey and game species as well as its potential use as sentinels for tularaemia, are briefly reviewed.

Secondly, a review is made on rabbit haemorrhagic disease (RHD), a highly contagious and fatal infection, which affects both wild and domestic rabbits and has been responsible for the dramatic reduction in wild rabbit populations as well as for substantial losses in the rabbit industry. This review addresses the disease brief history, aetiologic agent, virus life cycle, pathogeny, clinical signs and lesions, laboratorial diagnosis, eco-epidemiology, genetic diversity, host virus co-evolution, disease prevention and control and immunoprofilaxy. The topics directly related with the studies on RHD presented in this thesis, although mentioned in *General Introduction*, are approached in specific reviews that precede each set of studies in the *Results* Section.

Finally, the most significant aspects on tularaemia, an emergent/re-emergent zoonosis for which wild leporids are considered main reservoirs, are presented in the form of a review article, already published in an international refereed scientific journal, which includes the disease history, the aetiologic agent (*Francisella tularensis*) microbiology and phylogeography, epidemiology, life cycle, immunopathogenesis, clinical manifestations, laboratory diagnosis, treatment and vaccination. A brief update regarding this infection is given after the article is presented. Nevertheless, the update of the topics directly related with the studies on tularaemia presented in this thesis, are also addressed in specific introductions preceding each study.

1.1. The European wild rabbit (*Oryctolagus cuniculus*)

The European wild rabbit *O. cuniculus* (Linnaeus, 1758) is a high-profile prey native to the Iberian Peninsula and one of the rare mammals originally domesticated in Western Europe (Monnerot et al., 1994). *O. cuniculus* is the sole representative of its genus and belongs to the *Leporidae* family of the order *Lagomorpha* (Matthee et al., 2004).

1.1.1. Brief evolutionary history

The origins of *O. cuniculus* lay in the Iberian Peninsula. Southern Spain has been considered the most probable geographic origin of the *Oryctolagus* genus (Ferrand and Branco, 2007) as a rabbit fossil with 6.5 million years (my) was discovered in the region of Granada (Andalusia, Spain) (Lopez-Martinez, 1989)(Monnerot et al., 1994)(Ferrand and Branco, 2007). *O. cuniculus* appeared 900,000 years ago in Southern Spain and much later in Southern France (≈300,000-500,000 years ago) (Ferrand and Branco, 2007)(Queney et al., 2001), after crossing the Pyrenean barrier. In Portugal, the rabbit was the most abundant mammal in the Tejo and Sado valleys Mesolithic sites (Lentacker, 1986)(Arnaud, 1987) and bone ornaments in the shape of rabbits were found in the country dating back to the second half of the 4th millennium B.C. (Leisner, 1983)(Ferrand and Branco, 2007).
The biogeography of the rabbit in its original distribution area was most likely strongly affected by the later glacial events (that took place until 12,000 years ago) (Monnerot et al., 1994) and more recently, by Man. Human interference affected the dispersion of this species through transportation, eventually accompanied by domestication and restocking (Monnerot et al., 1994).

1.1.1.1. Geographic expansion

O. cuniculus geographical expansion has been one of the most remarkable mammal expansions' (Ferrand and Branco, 2007). From the Iberian Peninsula and southern France, the rabbit was able to disseminate to other locations as consequence of environmental factors and human interference (Callou, 1995).
From the Pleistocene to the Neolithic the species geographic distribution was strictly related to climate and vegetation (Callou, 1995). In this period, the rabbit presence was extremely marked in Spain (except the north regions of Alava, Guipuzcoa and Navarre) and South of France (except west of the Garonne river). The species was also able to reach the North of France, although limited to the south of the Loire River (Callou, 1995).

From the Bronze Age until the Vth century A.D, the rabbit preserved his distribution in the continent but was introduced in some of the Mediterranean Islands, such as the Balearic Islands (Majorca (1100 B.C.), Menorca (XIV-XIII centuries B.C.), Ibiza and Formentera (from the VIII century B.C. onwards) (Vigne and Alcover, 1985)) and Zembra (already present in the II or III century B.C. (Vigne, 1988)) (Callou, 1995). There are no evidences of the rabbit presence in Corsica, Sardinia or Sicily in this period (Callou, 1995). The first domestication attempts of the species were carried out by the Romains at the end of this period (Callou, 1995).

The Middle Age represented the rabbit most important expansion epoch (Callou, 1995). After domestication, Man acted as the main vector of the rabbit diffusion (Callou, 1995). During this period, the species was exported to central and northern Europe, the British Islands (XII century A.D.) an several other locations in the world (Callou, 1995)(Queney et al., 2001)(Branco and Ferrand, 2002).

The rabbit very successful process of transport and colonization began with Mediterranean navigators (Flux and Fullagar, 1992)(Ferrand and Branco, 2007). By the XVIth century, the species had been already introduced in several regions of Western Europe, such as Germany (1407, lake Schwerin island) and Hungry, and in the Atlantic islands of Porto Santo (1418) and Azores (in the XVth century, by Portuguese navigators) (Callou, 1995)(van der Loo et al., 1999)(Esteves et al., 2004)(Ferrand, 2008)(Esteves et al., 2014). Rabbit introductions in northern Africa occurred at approximately 3,000 years ago (Dobson, 1998)(Queney et al., 2001). The first attempts to introduce the rabbit in Australia date back to the end of the XVIIIth century (years of 1787 and 1791) but were only effective in 1859. Latter, the rabbit was introduced in the islands of New Zealand (in 1864) and Kerguelen (in 1874) (Callou, 1995). Chile imported the species in 1910 (Angerman, 1974)(Callou, 1995).

Currently, the rabbit is considered a successful colonizer with a worldwide distribution, from subtropical to sub-Antarctic climates (**Figure 1**). *O. cuniculus* is present in Australia and New Zealand, South America, and in more than 800 islands throughout the world (Flux and Fullagar, 1992)(Queney et al., 2001)(Ferrand and Branco, 2007). The species occurs in a remarkable diversity of ecological contexts where it is regarded as a pest, a biological invader or rather a key species upon which a variety of threatened predators depend on to survive (reviewed in (Ferrand and Branco, 2007)).

The human perception of the *O cuniculus* species changed with the expansion of the rabbit territory. From strictly wild, the rabbit became also “wild-domestic” and finally “wild” and “domestic” (Callou, 1995).

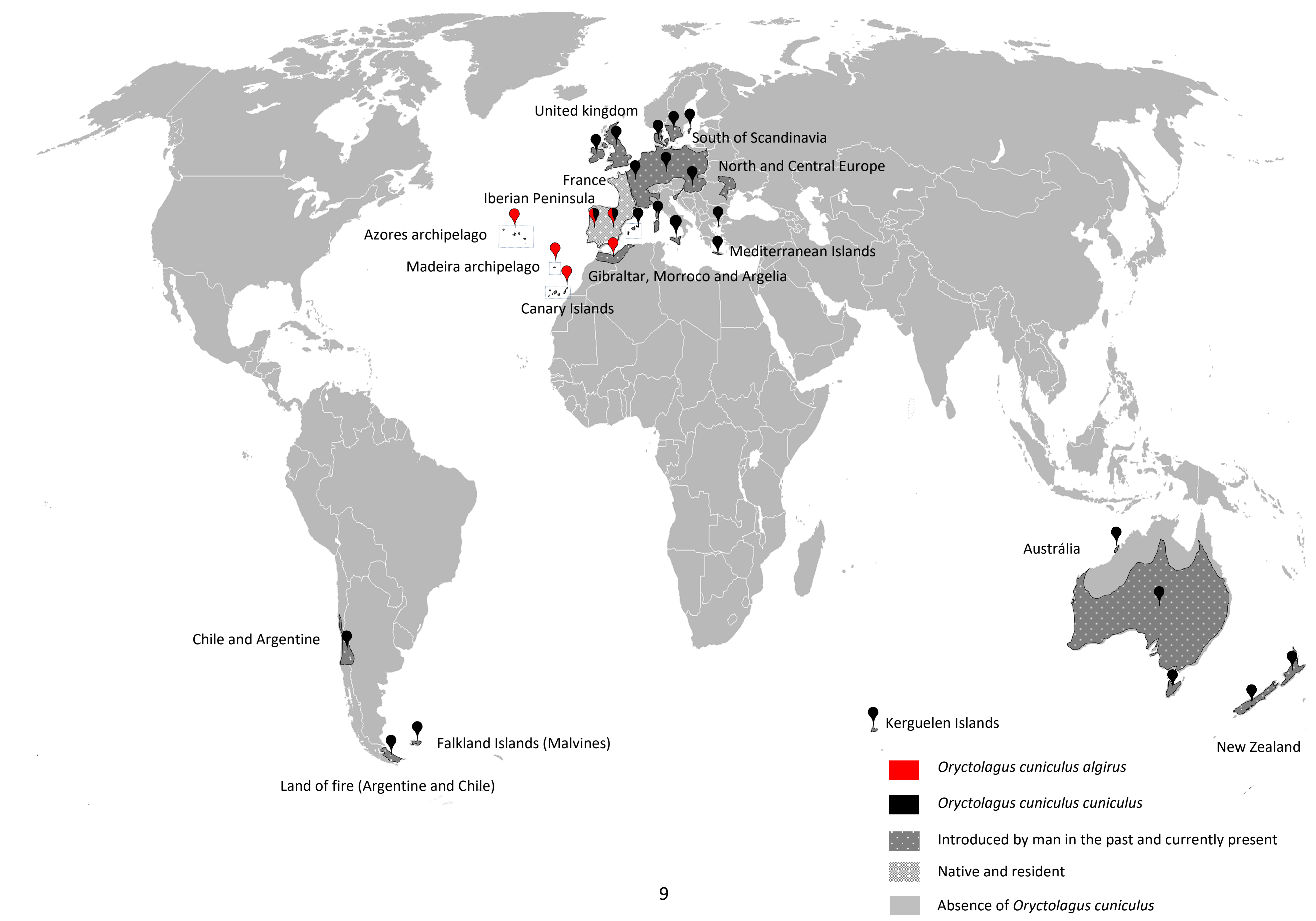


Figure 1. ***Oryctolagus cuniculus*** **world distribution.** This map was based on the IUCN map for the species world distribution and edited according to (Callou, 1995) and (Ferrand and Branco, 2007).

1.1.1.2. Genetic diversity

Two *O. cuniculus* subspecies are recognized, specifically, the *O. cuniculus algirus* (**Figure 2 A and B**) and the *O. cuniculus cuniculus* (Lopez Martinez, 1989). Both are brown/grey in colour but the *O. c. algirus* subspecies is smaller (adults medium weight of 1.100 Kg and maximum weight of 1.400 Kg) (Soriguer, 1980)(Ferreira and Ferreira, 2014) than *O. c. cuniculus* (adults maximum weight of 2.00 Kg) (Gibb, 1990). There are no distinct gender differences in both subspecies (Gibb et al., 1985)(Ferreira and Ferreira, 2014).

Figure 2. (A) and (B) *Oryctolagus cuniculus algirus*, the most abundant subspecies in Portugal (photos taken during wild rabbit translocations in Portugal and kindly relinquished by Doctor Margarida Ramada de Melo).

O. c. algirus is endemic to the south-western Iberian Peninsula, and present with a more restricted distribution. It is also present in the North Africa and Atlantic islands (Madeira, Azores and Canaries). In contrast, *O. c. cuniculus* inhabits the north-eastern part of the Iberian Peninsula and is widespread in the other locations where the species occurs (Branco et al., 2000)(Ferrand and Branco, 2007).

The fact that *O. c. algirus* populations are characterized by a higher genetic variability than *O. c. cuniculus* populations (Ferrand and Branco, 2007) is compatible with the species Iberian origin and suggests that the separation and long geographic isolation of both subspecies may have generated an asymmetrical division of genetic diversity within *O. cuniculus* (Ferrand and Branco, 2007). Solid evidences of the existence of two major population groups that have been evolving independently for a long period of time, conforming with the modern subspecies concept (Avise and Hamrick, 1996), were provided by the genetic data from Ferrand and Blanco (2007) study as well as evidences of immunoglobulin polymorphism (van der Loo et al., 1991)(van der Loo et al., 1999) and mtDNA variation (Biju-Duval et al., 1991)(Monnerot et al., 1994). In fact, immunoglobulin allotypes

with genetic polymorphism found in IgKC1, IgVH1 and IgGCH2 *loci* where demonstrated by serologic studies (van der Loo, 1987)(van der Loo et al., 1987)(Cazenave et al., 1987). While a high degree of genetic identity was found between domestic and wild rabbit populations from central Europe and Australia, as no new alleles were described and both allelic distribution profiles were very similar (Curtain et al., 1973)(van der Loo 1987)(van der Loo et al., 1987), an entirely different scenario was revealed when this investigation was extended to wild rabbit populations from the Iberia. In the latter populations, the *loci* were found to harbour much higher variation with seven to eight new alleles in IgKC1 (Cazenave et al., 1987)(van der Loo et al. 1991) and at least 10 new alleles in IgVH1 (Cazenave et al., 1987), with no polymorphism for IgGCH2 (Cazenave et al., 1987)(van der Loo et al., 1991). These two distinct scenarios regarding the immunoglobulin polymorphism profile support the rabbit Iberian origin and are in accordance with two divergent genetic entities.

Likewise, when Biju-Duval et al. (1991) and later Monnerot et al. (1994) investigated patterns of sequence variation in rabbit mitochondrial DNA (mtDNA), the occurrence of two very divergent mtDNA lineages was exposed. One (lineage A) was circumscribed to southwest Iberia and Azores islands, while the other (lineage B) occurred in northern Spain, France, England, the rest of Europe, Australia and in all domestic breeds (Biju-Duval et al., 1991)(Monnerot et al., 1994). In the broad survey of Iberian wild rabbit populations carried out by Blanco et al., (2000), those two mtDNA lineages were shown to be essentially allopatric, with a very limited overlap along a northwest southeast gradient that divides the Peninsula. Furthermore, Iberian rabbits were shown to display high levels of inter- and intra-population variability (Branco et al., 2000) in contrast with rabbits from France, which do not express intra-population polymorphism (Monnerot et al., 1994). These data suggest that south-western Iberian rabbit populations are older and that occupation of southern France occurred more recently, corroborating the rabbit Iberian origin (Ferrand and Branco, 2007).

In accordance, Ferrand and Blanco (2007) genetic analysis indicated that an ancient split, supported by a high bootstrap value (99% to 100%), separated the south-western Iberian and Azorean Islands rabbit populations from all others, including domestic breeds, corresponding most probably to *O.c. algirus* and *O.c. cuniculus* emergence, respectively. The high degree of genetic differentiation between the two subspecies allowed the reconstruction of rabbit geographical expansion. While France, Britain and other European countries, as well as Australia, were colonized by *O. c. cuniculus*, from which domestic breeds are exclusively derived, the Azorean island populations represent an

expansion of the *O. c. algirus* and show evidence of a strong bottleneck effect, since their genetic diversity represents a subset of the genetic diversity found in the Iberian Peninsula population.

In brief, it is suggested that *O. c. algirus* (lineage A) and *O. c. cuniculus* (lineage B) diverged following isolation in two glacial refugia in the Iberian Peninsula south-western and north-eastern extremes, respectively, most likely during the Quaternary paleoclimatic oscillations. After a climatic improvement, they expanded their ranges to interact in a secondary contact zone along a northwest–southeast axis where they hybridized (Branco, et al. 2000)(Branco et al., 2002)(Ferrand and Branco, 2007). The Iberian Peninsula is the only region in the world where the two formally recognized subspecies, *O. c. algirus* and *O. c. cuniculus* currently co-exist and hybridize in natural conditions (**Figure 3**)(Ferrand, 2008)(Ferreira, 2012)(Alda and Doadrio, 2014).

In the Alda and Doadrio (2014) study, the genetic diversity found in the hybrid zone was higher than the one observed in the parental populations. Forty-nine alleles were exclusively observed in this region, a number superior to the sum of the parental alleles. This could be the result of alleles' recombination from the parental lineages or of the unbalanced genetic contribution of each rabbit lineage.

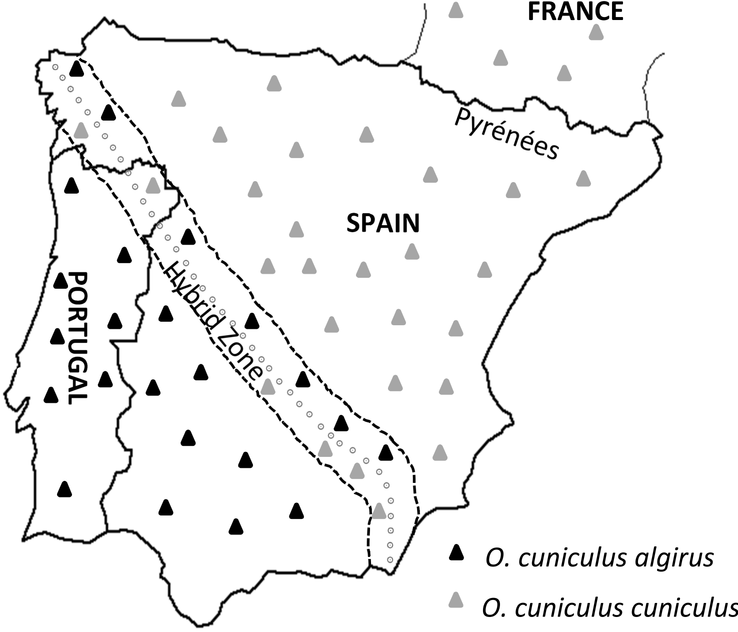


Figure 3. *O. cuniculus* subspecies distribution in the Iberian Peninsula. The subspecies *O. c. algirus* and *O. c. cuniculus* are essentially allopatric, with a very limited overlap along a north-west/south-east gradient that divides the peninsula (the hybrid zone) (adapted from (Branco et al., 2000)(Alda and Doadrio, 2014)).

1.1.2. Habitat

Rabbits select their habitat based on two main drivers, food provision and predation avoidance (Dellafiore et al., 2014). Favourable habitats for rabbits include herbaceous patches, providing adequate food resources and scrubland cover or warrens, offering protection against predators (Beja et al., 2007). Wild rabbits prefer scrubs with a low density of woody vegetation at the ground level, but with a dense overhead cover (Beja et al., 2007). The herbaceous layer also offers cover, by grasses under the scrub, and forbs, at the edge of the scrub (Beja et al., 2007).

Habitat deterioration and/or fragmentation have heavily contributed for wild rabbit's decline throughout the Iberian Peninsula (Moreno and Villafuerte, 1995), playing a cumulative role with the incidence of viral diseases and predation (Trout and Tittensor, 1989)(Ferreira and Alves, 2009). In the European Mediterranean region, the abandonment of agricultural land and its subsequent occupation by scrubland and woodland has resulted in habitat losses for species associated with early-successional vegetation and edge habitats, which had benefited from the mosaic structure shaped by the traditional management of Mediterranean landscapes (Beja et al., 2007)(Delibes-Mateos et al., 2010)(Ferreira and Delibes-Mateos, 2010).

Rabbits' distribution in different landscapes is influenced by topography (rabbits are more abundant in continuous areas than in fragmented ones (Virgós et al., 2003), soil hardness, climatological conditions and agricultural practices (types of crops) as well as by some agricultural landscape components (Calvete et al., 2004a). Vegetative cover has been positively associated with rabbit abundance and scrub cover in naturally vegetated sites within agricultural landscapes is important in maintaining rabbit abundance (Calvete et al., 2004a).

The major part of the total variability found between and within habitats in the Iberian Peninsula was attributed to differences between *O. c. algirus* and *O c. cuniculus*, (Ferrand and Blanco, 2007).

Figure 4. Mosaic structure of the landscape created by agricultural management (photo taken by Doctor Fílipe Barroso and kindly relinquished by the Instituto de Ciências Agrárias e Ambientais Mediterrânicas (ICAAM)).

1.1.3. Diet

Rabbits are generalist herbivores consuming a large spectrum of plant species (Ferreira and Alves, 2009). While preferring grasses (Martins et al., 2002), this species explores different vegetation strata and adapts to the quantity and quality of available resources (Ferreira and Alves, 2009).

There is a seasonal pattern of rabbit abundance in the Iberian Peninsula associated with food resources (Villafuerte et al., 1997)(Ferreira and Alves, 2009). During the winter, rabbits feed mainly on herbs but in particularly critical periods (summer), a displacement of rabbit's diet is observed (Martins et al., 2002)(Beja et al., 2007), with higher consumption of leaves and scrubs seeds (Beja et al., 2007) or shrub-like dicotyledons such as gum cistus (*Cistus ladanifer*), a low nutritional value plant of difficult digestion due to its high fibre content (Martins et al., 2002)(Beja et al., 2007). The decrease in rabbits' abundance in summer is usually associated to the poverty of food resources (Villafuerte et al., 1997)(Ferreira and Alves, 2009). The species' reproductive behaviour and survival may also be endangered during the hot dry season since a poor physical condition increases predation risk and the individuals susceptibility to epizootic events (Ferreira and Alves, 2009).

1.1.4. Population dynamics

The European rabbit is highly prolific and can reproduce opportunistically at any season, which contributes to its success as a colonist (Gibb, 1990). Wild rabbits' population dynamics is more sensitive to changes in mortality (particularly juvenile mortality) than in fecundity (Smith and Trout, 1994)(Smith, 1997)(Calvete et al., 2005). In Mediterranean areas, and in particular in the Iberian Peninsula, the distribution and abundance of wild rabbit populations is highly variable between and within habitats (Virgós et al., 2003)(Calvete and Estrada, 2004)(Calvete, 2006). High intra- and inter-year variability within populations is also observed (Calvete, 2006).

The onset of rabbit reproduction, and hence its influence on rabbit density, is not merely related with an increase in food quality but also with the availability of high quality food (Villafuerte et al., 1997).

In stable wild populations, the high reproductive rate must be balanced by a high rate of juvenile mortality. More than 80% of the young born may be killed by predators, either in the nest or within the first two weeks of life (Gibb, 1990). Also, competition for refuge, feeding areas and warrens can increase juvenile mortality, when carrying capacity is reached (Myers et al., 1994)(Calvete, 2006). Regarding adult rabbits, the annual mortality rates are highly variable usually ranging from 20% to 80% (Gibb, 1990)(Calvete et al., 2005).

Despite these aspects, the main factor affecting the structure of wild rabbit populations are epizootic diseases (Monnerot et al., 1994), namely those induced by rabbit haemorrhagic disease virus (RHDV) (Liu et al., 1984) and Myxoma virus (Aragão, 1927)(Fenner and Ratcliffe, 1965)(Sanarelli, 1898). RHD has been responsible for local extinctions of *O. cuniculus* (Ferreira e Alves, 2009).

1.1.5. Rabbit population trends

Until recently, the wild rabbit has been always abundant in the Iberia (Ferreira and Delibes-Mateos, 2010). Yet, historically, there has been a negative trend regarding rabbit populations in the Iberia Peninsula, mainly as a consequence of habitat loss and viral diseases (Delibes-Mateos et al., 2009)(Ferreira and Delibes-Mateos, 2010). Other scarcely studied factors may have contributed to the long-term rabbit decline, including unsustainable hunting, predation or climate change (Ferreira and Delibes-Mateos, 2010).

Wild rabbit decline in the Iberian Peninsula was already ongoing in the first half of the XXth century, before viral diseases emerged, possibly as a consequence of habitat loss and fragmentation (Ales et al., 1992)(Ferreira and Delibes-Mateos, 2010). The latter, resulted from changes in the agrarian structure, and agriculture and livestock farming intensification, along with the under-utilization of the traditional farming practices in rural areas (Ferreira and Delibes-Mateos, 2010). These processes originated the loss of the wild rabbit's most preferred habitat, the Mediterranean mosaics that characterized the traditional Iberian agricultural landscapes (Ales et al., 1992)(Beja et al., 2007)(Delibes-Mateos et al., 2010)(Ferreira and Delibes-Mateos, 2010).

Wild rabbit populations downsizing in the Iberian Peninsula was greatly accelerated by the arrival of myxomatosis during the 1950's (Ferreira and Delibes-Mateos, 2010) and rabbit haemorrhagic disease in 1989 (Villafuerte et al., 1995).

Despite rabbit populations in their natural ranges have declined dramatically over the past century (>90%) (Delibes-Mateos et al., 2009)(Ferreira and Delibes-Mateos, 2010), positive trends were recorded in species-friendly habitats, characterized by soft soils and sparse Mediterranean scrublands interspersed with good pastures and/or crops (Calavete et al., 2006)(Delibes-Mateos et al., 2009)(Ferreira, 2012).

Presently, most wild rabbit population are still declining in different regions of the Iberian Peninsula and negative trends in rabbit numbers have been reported both in Portugal and Spain (Delibes-Mateos et al., 2014)(Instituto da Conservação da Natureza e das Florestas, IP (ICNF)), mostly in consequence of the emergence of the new rabbit haemorrhagic virus 2 (RHDV2) in both Spain (Dalton et al., 2012) and Portugal (Abrantes et al., 2013). RHDV2 have been threatening the survival of *O. cuniculus algirus* as well as of the predators that feed on the rabbit (Abrantes et al., 2013)(Delibes-Mateos et al., 2014). Furthermore, the wild rabbit downsizing has been interfering with other trophic chains were the predators find alternative preys for their survival, such as the Sardão lizard (*Lacerta lepida*) (Eng.º Ricardo Paiva, Instituto Nacional de Investigação Agrária e Veterinária (INIAV), *personal communication*).

Different management techniques are employed to reverse this downsizing, including adjusting hunting pressure, predator control, habitat management, restocking and rabbit vaccination (Ferreira and Delibes-Mateos, 2010). It is interesting to notice that rabbits have showed to recover better in hunting estates where various game management strategies have been applied, both regularly and simultaneously (such as low hunting pressure, predator control or habitat management)(Delibes-Mateos et al., 2009).

The importance of management strategies to enhance the wild rabbit populations is specifically addressed in Chapter IV ("RHD eco-epidemiology") point "1.6.4. Habitat management".

1.1.5.1. IUCN red list category

As a result of the European rabbit progressive decline in the Iberian Peninsula, the species has been classified as a *Near-Threatened* and *Vulnerable* species by the IUCN criteria since 2008 (Smith and Boyer, 2008), figuring in the Red List of Vertebrates of Portugal (Cabral et al., 2005) and Spain (Ferreira, 2012).

1.1.5.2. Hunting records to estimate rabbit population abundance

Hunting records can be used to estimate population abundance although not necessarily accurately reflecting species abundance, given that they can be misleading if hunting effort is not controlled (Delibes-Mateos et al., 2009)(Ferreira and Delibes-Mateos, 2010).

Based in a 11 year period that ranged from 2005 to 2016, a negative trend regarding wild rabbit populations has also been observed in Portugal and further aggravated by RHDV2 emergence in the country in 2012 (Abrantes et al., 2013)(Duarte et al., 2015b). The number of specimens hunted in the cinegetic period 2011/2012 was 688, 759 and, since then, this number steadily decreased until the present (Instituto da Consevação da Natureza e das Florestas, IP, ICNF). The number of wild rabbits hunted during the cinegetic period of 2015/2016 was 267, 479, less than half of the number of wild rabbits registered in the homologous period of 2011/2012 (**Table 1**) (Instituto da Conservação da Natureza e das Florestas, IP, (ICNF)

Table 1. Wild rabbit (*O. cuniculus*) specimens hunted in Portugal mainland between the years 2005 and 2016, during the cinegetic period (from September 1st to December 31st). Data was provided by Dr. Gonçalo Lopes from the Instituto da Conservação da Natureza e das Florestas, IP (ICNF). To compare with the homologous data regarding the Iberian hares (*L. granatensis*) hunted in Portugal during the same period Cf. Table 10, page 35.

Species		**Cinegetic period**										
		2005/6	**2006/7**	**2007/8**	**2008/9**	**2009/10**	**2010/11**	**2011/12**	**2012/13**	**2013/14**	**2014/15**	**2015/16**
Wild rabbit (*O. cuniculus*)	Nº of hunted specimens	687, 553	559, 121	531, 622	592, 460	659, 081	653, 181	688, 759	630, 313	332, 907	322, 118	267, 479

1.1.6. The effect of rabbit population dynamics on RHD impact

This theme is specifically addressed in Chapter IV ("RHD eco-epidemiology"), point "1.5 The effect of rabbit population dynamics on RHD impact".

1.1.7. Importance of the European rabbit in the Iberian Peninsula

Wild rabbit has a wide distribution in the Iberian Peninsula and is present in diverse environments, from Mediterranean scrublands to agro ecosystems, mountainous areas, as well as coastal and dunes (reviewed in Dellafiore et al., 2014).

In the Iberia, *O. cuniculus* accumulates paradoxical labels (Ferreira, 2012) and is the centre of serious management and conservation issues (Delibes-Mateos et al., 2011). In fact, the heterogeneous spatial distribution of the species allows it to be considered concurrently absent and a pest, in two locations just a few kilometres apart (Ferreira and Delibes-Mateos, 2010)(Ferreira, 2012). Furthermore, while benefiting from local threatened conservation status, it is also one of the most important small-game species (Ferreira, 2012)(Ferreira and Ferreira, 2014). The multiple *O. cuniculus* roles in the Iberian Peninsula are presented below.

1.1.7.1. A keystone species and "engineer" in the Mediterranean ecosystem of the Iberian Peninsula

Wild rabbits are multifunctional keystone species in the Mediterranean ecosystem of the Iberian Peninsula (Delibes-Mateos et al., 2008). Here, they are considered as "ecosystem engineers" given their effect on landscape structure (Gálvez et al., 2008)(Delibes-Mateos et al., 2008)(Ferreira and Alves, 2009) and potentially favourable environments can be perpetuated by the species when it reaches certain abundance levels (Ferreira and Alves, 2009). Wild rabbits conspicuously alter plant species composition and vegetation structure through grazing disturbance and seed dispersal, creating open areas and preserving plant species diversity (Delibes-Mateos et al., 2008). Moreover, rabbit latrines contribute to soil fertility and plant growth and provide new feeding resources for many invertebrate species (Delibes-Mateos et al, 2008). Rabbit burrows also offer nest sites and shelter for vertebrates and invertebrates (Delibes-Mateos et al., 2008). In addition, rabbits disappearance in this ecosystem would precipitate further species extinctions (Mills et al., 1993)(Ferreira, 2012), as they serve as prey for several predator species which depend on high rabbit densities for survival (Calvete and Estrada, 2004)(Delibes-Mateos et al., 2008). Those include

the *Endangered* Iberian lynx (*Lynx pardinus*) and the *Vulnerable* Iberian Imperial Eagle (*Aquila adalberti*) (Calvete et al., 2005)(Delibes-Mateos et al., 2008).

Rabbits importance in the Mediterranean ecosystem of the Iberian Peninsula is such that some researchers sustain it should be entitled "the rabbit's ecosystem" (Delibes-Mateos et al., 2008).

1.1.7.2. An important game species

Apart from their central role in nature, *O. cuniculus* has also an economic and cultural function in the Mediterranean countries, such as Spain and Portugal, regarding small game hunting (Virgós et al., 2007)(Ferreira and Ferreira, 2014). Rabbits are coveted prey with high social value in the Iberia providing an important source of income in rural areas (Beja et al., 2007).

The Portuguese national territory is subordinated to the legal hunting regime (Decree-Law No. 2/2011 establishing the legal regime for the exploitation and conservation of hunting resources). In the cinegetic period of 2016/2017 the hunting market included an estimated number of 250, 000 hunters holding licenses (**Table 2**) and near 2, 000 non residents hunting licenses were emitted for the referred period (**Table 3**). There are 4, 900 shooting areas in the country including associative, touristic, municipal and national hunting areas (**Table 4**). Paixão et al. (2009) refer a revenue of 365 million euros generated by hunting activities each year in Portugal, derived from hunting licenses, number of animals shot for meat consumption and restocking actions.

Table 2. Number of holders of hunting licenses from 2011 to 2016. Data kindly provided by Instituto da Conservação da Natureza e das Florestas, IP (ICNF).

Year	Nº of holders of hunting licenses
2011	286, 917 (*)
2012	282, 870 (*)
2013	279, 498
2014	272, 361
2015	263, 009
2016	256, 179

(*) Estimated numbers

Table 3. Number of hunting licenses per type emitted in Portugal from the cinegetic period of 2008/2009 to the homologous period of 2016/2017. Data kindly provided by Instituto da Conservação da Natureza e das Florestas, IP (ICNF).

	N.º of licenses emitted per type		
Cinegetic period	**National**	**Regional**	**Non residents**
2008/2009	66, 336	85, 052	1, 125
2009/2010	62, 105	82, 024	1, 022
2010/2011	59, 368	80, 219	1, 074
2011/2012	55, 052	80, 501	1, 160
2012/2013	49, 924	81, 738	1, 137
2013/2014	46, 049	77, 514	1, 292
2014/2015	43, 184	75, 488	1, 274
2015/2016	41, 750	72, 657	1, 622
2016/2017	41, 724	78, 071	1, 811

Table 4. Number, type and area in hectares (ha) of the cinegetic zones in Portugal. Data kindly provided by Instituto da Conservação da Natureza e das Florestas, IP (ICNF).

	Hunting areas per type									
	Associative (ZCA)		Touristic (ZCT)		Municipal (ZCM)		National (ZCN)		Total	
Cinegetic region (category)	Nº	Area (ha)	Nº	Area (ha)	Nº	Area (ha)	Nº	Area (ha)	Nº	Area (ha)
1ª	352	653, 844	20	21, 033	276	942, 989	1	21, 190	649	1, 639, 056
1ª/2ª	4				3				7	0
2ª	431	666, 178	113	114, 029	287	1, 007, 042	1	10, 851	832	1, 798, 100
2ª/3ª			1		2				3	0
3ª	402	451, 492	163	181, 835	123	283, 725	2	6, 363	690	923, 415
3ª/4ª	15		12		3				30	0
4ª	1, 238	1, 127, 405	1, 010	918, 110	157	198, 904	1	5, 267	2, 406	2, 249, 686
4ª/5ª	11		1		1				13	0
5ª	191	198, 660	36	37, 727	43	144, 166			270	380, 553
Total	2, 644	3, 097, 579	1, 356	1, 272, 734	895	2, 576, 826	5	43, 671	4, 900	6, 990, 810

ZCA – Zona de caça associativa; ZCT – Zona de caça turistica; ZCM – Zona de caça municipal; ZCN – Zona de caça nacional

Currently, wild rabbits can be hunted during the autumn–winter season, which begins September 1st and ends late December (31st) (Instituto da Consevação da Natureza e das Florestas, IP, ICNF).
Using a simplistic model (which did not include a stochastic variation of natural populations), Calvete et al. (2005) showed that the impact of hunting on population dynamics is highly determined by hunting selection, which can be age- and sex-biased, and may vary throughout the year (Calvete et al., 2005). Autumn–winter hunting occurs at the start of the reproductive season (Calvete et al., 2005) and may potentially lead to reductions in the size of the wild rabbit stable populations (Kokko and Lindstrom, 1998). Nonetheless, Calvete et al. (2005) concluded that this period was the most conservative option for the harvesting of wild rabbit populations. To reduce the hunting impact, Calvete et al. (2005) also suggested the shortening of the hunting season by finishing it at the end of autumn, avoiding hunting during the winter, when the proportion of pregnant females is higher.

1.1.7.3. An agricultural pest

Regardless of the general negative trend of rabbit populations in Iberia, the "agricultural pest" label has recently been added to the list of roles played by the rabbit in the Iberia (Ferreira, 2012). This tag was justified by the putative damages rabbits cause to crops in certain areas (Ferreira and Delibes-Mateos, 2010)(Ferreira, 2012). Nevertheless, considering rabbits as a pest appears to be more related with a change in the perception of damages, both by hunters and farmers, than with actual demographic changes (Ferreira and Delibes-Mateos, 2010)(Ferreira, 2012).

1.1.8. The economic role of rabbit in the industry

The economic importance of the rabbit, mainly in its domestic type, is extensible to the meat and fur industries.
In several countries, the rabbit industry was also severely affected by RHDV2 emergence, including France (Le Gall-Reculé et al., 2011a), Spain (Dalton et al., 2012) and Portugal (Carvalho et al., 2017a)(Carvalho et al., 2017b).

1.1.8.1. Relevance of the rabbit meat industry sector in Portugal

The following information on the national rabbit meat industry was kindly given by Eng.º António Fernandes (ASPOC - Associação Portuguesa de Cunicultura, *personal communication*). Data are from the Instituto Nacional de Estatística (INE) processed by ASPOC.

In Portugal, the total number of industrial meat rabbitries (CAE – Código de Actividades Económicas) has slightly expanded in the last years (**Table 5**). Most of these corporations have less than 10 workers (2 workers on average), although the total number of workers has also increased (**Table 5**). Following this positive trend, the rabbit meat industry turnover in our country slightly augmented in the last years, despite a little decrease in 2015 (**Table 5**), related in part to the sharp reduction of the price of live rabbits that year.

Table 5. Data on the industrial meat rabbitry sector in Portugal from 2012 to 2015. The total number of corporations (CAEs) and workers is provided as well as the global turnover (in euros) in the period considered. This information was kindly provided by Eng.º António Fernandes (source: data from Informação empresarial simplificada (IES) - INE - processed by ASPOC).

	Year			
	2012	**2013**	**2014**	**2015**
Number of industrial meat rabbitries (CAE)	97	112	114	125
Number of workers in the sector	186	202	211	229
Global turnover (euros)	16, 897, 457	18, 723, 153	18, 116, 174	17, 589, 838

In 2014, meat rabbits were the 3rd species category with the highest slaughter numbers in Portugal after poultry and swine. Data on the total number of slaughtered meat rabbits in Portugal from 2011 to 2016 is provided in **Table 6**.

Table 6. Data on the total number of slaughtered meat rabbits in Portugal from 2011 to 2016, by number of animals (x 1000) and carcass weight (t - tonnes). This information was kindly provided by Eng.º António Fernandes (source: data from INE processed by ASPOC).

	Year	Numbers of rabbits slaughtered
Number of animals (x 1000)	2011	5, 416
	2012	5, 471
	2013	5, 206
	2014	5, 364
	2015	4, 860
	2016	4, 247
Carcass weight (t)	2011	6, 747
	2012	7, 138
	2013	6, 485
	2014	6, 762
	2015	5, 952
	2016	5, 199

In 2014, the country produced a total of 5, 430, 000 rabbits, from which 4, 800, 000 were slaughtered in the national territory. Additionally, 590,000 live rabbits imported from Spain were slaughtered in Portugal.

A total of 5, 000 rabbits *per* week originate from commercial trades with Spain. In 2015, it was estimated 1, 220, 000 live rabbits and 231, 000 Kg of rabbit meat were exported to Spain. Data on the production, importation and exportation of live meat rabbits and on the importation and exportation of rabbit meat from 2011 to 2016 in Portugal is given in **Table 7**.

The national production of rabbit meat in 2015 was estimated in 11.260 tonnes, generating an estimated turnover of approximately 17.6 million euros.

Table 7. Data on the production, importation and exportation of live meat rabbits and on the importation and exportation of rabbit meat from 2011 to 2016 in Portugal. This information was kindly provided by Eng.º António Fernandes (source: data from INE processed by ASPOC).

		Meat rabbits			Rabbit meat	
	year	**Production**	**Importation**	**Exportation**	**Importation**	**Exportation**
Number of animals (x 1000)	2011	5, 416	-	-	-	-
	2012	5, 471	-	-	-	-
	2013	5, 573	949	1, 316	-	-
	2014	6, 064	736	1, 437	-	-
	2015	6, 068	592	1, 800	-	-
	2015	6, 170	373	2, 296	-	-
Carcass weight (t)	2011	6, 747	-	-	-	-
	2012	7, 138	-	-	-	-
	2013	6, 941	1, 183	1, 639	3, 222	292
	2014	7, 645	928	1, 811	3, 567	514
	2015	7, 432	725	2, 205	3, 000	262
	2016	7, 552	456	2, 810	3, 664	116

In 2015, the rabbit meat consumption in Portugal was valued to 8, 766, 287 Kg (exclusively originated from industrial production), from which 5, 552, 000 Kg referred to rabbits that were slaughtered in Portugal and 304, 290 Kg referred to imported rabbits. The estimated consumption of rabbit meat in Portugal in 2015 was 0.84 Kg/ person/ year. Data on the consumption of rabbit meat in Portugal from 2011 to 2016 is given in **Table 8**.

Table 8. Data on the consumption of rabbit meat in Portugal from 2011 to 2016. This information was kindly provided by Eng.º António Fernandes (source: data from INE processed by ASPOC).

		Consumption of rabbit meat	
	Year	**Total (t)**	***Per capita* (Kg)**
Carcass weight	2011	6, 747	0. 67
	2012	7, 138	0.71
	2013	9, 414	0.94
	2014	9, 815	0.98
	2015	8, 690	0.87
	2016	8, 747	0.87

The self-supply rate on rabbit meat in Portugal from 2013 to 2016 is provided in **Table 9**.

Table 9. Portugal self-supply rate (rabbit meat), 2013 to 2016. This information was kindly provided by Eng.º António Fernandes (source: data from INE processed by ASPOC).

Year	Self-supply rate (%)
2011	-
2012	-
2013	73.7
2014	77.9
2015	85.5
2016	86.3

In 2015 the total production costs associated with the rabbit meat industries rose to 19.8 million euros, 60% (11.8 million euros) of which corresponded to expenses with feed. Considering the estimated rabbit meat industries turnover in Portugal in 2015 (17.6 million euros) and the estimated total production costs (19.8 million euros), there was a global financial loss of 2.2 million euros.

1.2. The Iberian hare (*Lepus granatensis*)

Hares belong to the *Leporidae* family of the order *Lagomorpha*. In the Iberian Peninsula, the genus *Lepus* is presently represented by three species: the Iberian hare (*Lepus granatensis* (Rosenhauer, 1856)), the Broom hare (*Lepus castroviejoi* (Palacios, 1976)) and the European brown hare (*Lepus europaeus* (Pallas, 1778)) (Duarte, 2000)(Gortazar et al., 2007)(Melo-Ferreira et al., 2007). Both *L. granatensis* and *L. castroviejoi* are endemic to the Iberian Peninsula (Duarte, 2000). Mitochondrial DNA (mtDNA) studies have identified lineages that are specific to each of these species (Pérez-Suárez et al., 1994)(Alves et al., 2003)(Melo-Ferreira et al., 2007). However, Alves et al. (2003) also detected haplotypes inherited from *Lepus timidus* (the mountain hare), now extinct from Iberia, in *L. granatensis* and *L. europaeus* specimens. *Lepus timidus* is an arctic-alpine species widely distributed in the Northern part of the Palaearctic region, and is found from the British Isles to the Russian Far East, as well as in some isolated populations in the Alps, Poland and Japan (Angerbjörn and Flux, 1995)(Melo-Ferreira et al., 2007). According to fossil records, *L. timidus* was the most common and widely distributed hare species in Europe during the last glacial periods (Lopez-Martinez, 1980) and mtDNA of *L. timidus* origin is widespread in the Iberian Peninsula (Melo-Ferreira et al., 2007). The *L. timidus* mtDNA introgression predominates in *L. granatensis* populations from the North, but becomes rarer towards the South, where it is absent. Moreover, it is almost fixed in Iberian *L. europaeus* and also present in *L. castroviejoi* (Melo-Ferreira et al., 2007).

1.2.1 Distribution

L. granatensis is widely distributed in the Iberia and its geographic range includes Portugal and nearly the entire extent of the Spanish territory (Alves et al., 2003)(Gortazar et al., 2007). Still, it is absent from Spanish northern regions, where *L. castroviejoi* (restricted to the Cantabrian Mountains) and *L. europaeus* (along the Pyrenees) are found (Alves et al., 2003)(Gortazar et al., 2007)(Melo-Ferreira et al., 2007) (**Figure 5**). These regions correspond to the geographic triangle that runs from the Atlantic coast along the Pyrenees to the Ebro delta, the *L. granatensis* southernmost limit in Europe (Alves et al., 2003)(Gortazar et al., 2007). *L. granatensis* and *L. europaeus* exist in parapatry in most Northern Spain provinces, while the Iberian hare inhabits the southern region, the brown hare is found in the north (Fernandez et al., 2004). *L. granatensis* is also present on Mallorca Island (Duarte, 2000) and has been introduced in southern France (Alves et al., 2003) (**Figure 6**).

According to the IUCN, the Iberian hare is abundant and common in the southern and central areas of its range (Mitchell-Jones et al., 1999)(Farfán et al, 2004) and the current population trend is considered stable. The species figures in the IUCN red list in the category of *Least Concern* (Smith and Johnston, 2008).

Although no major threats to *L. granatensis* have been pointed out (Smith and Johnston, 2008), a high hunting pressure, predation and diseases, such as the European brown hare syndrome virus (EBHSV) or tularaemia, could putatively represent important factors affecting the Iberian hare population dynamics (Duarte, 2000). Also, the use of rodenticides in agricultural lands and road traffic could be considered threats to the species (Purroy, 2011).

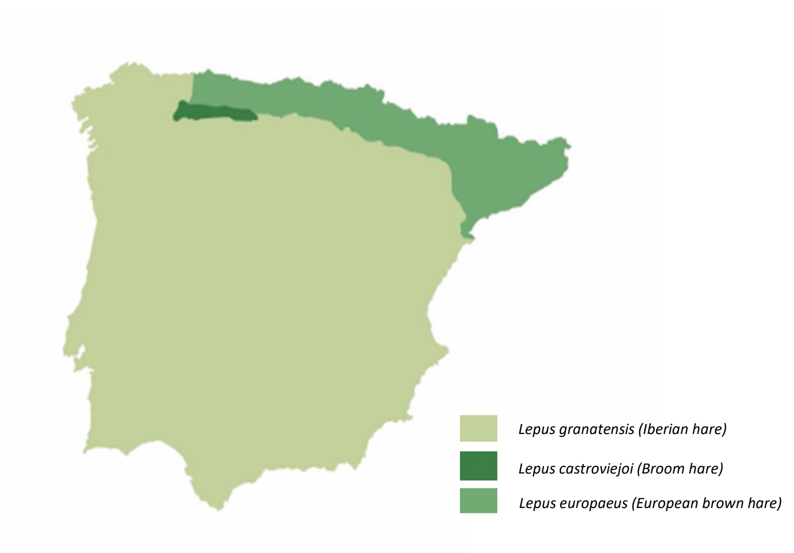


Figure 5. The distribution of the three hare species that can be found in the Iberian Peninsula. *Lepus granatensis* (gross distribution) is widely distributed in the Iberia (light green colour) while *Lepus castroviejoi* (dark green colour) is restricted to the Cantabrian Mountains and *Lepus europaeus* (medium green colour) occurs along the Pyrenees (adapted from (Campos et al., 2015)).

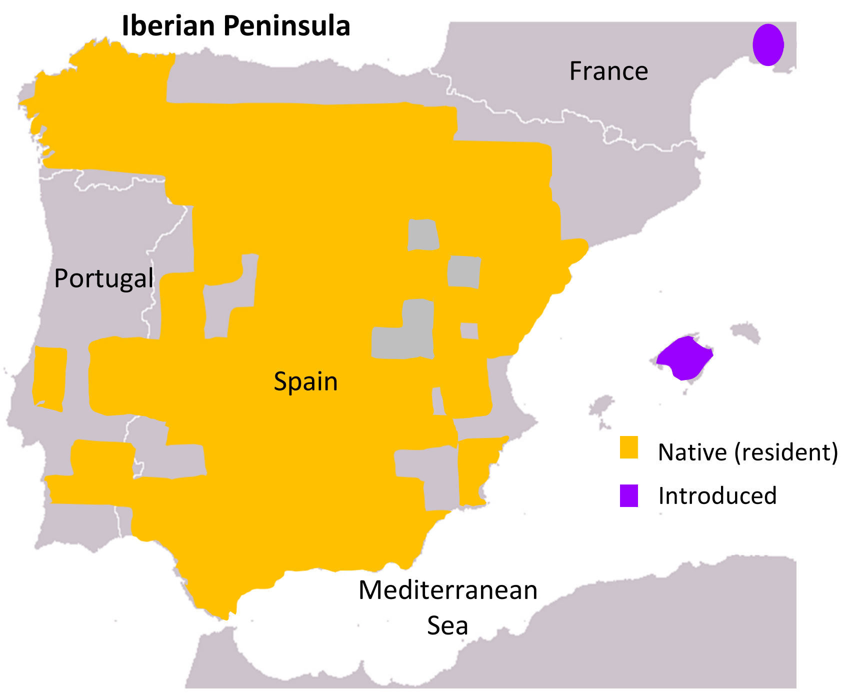


Figure 6. ***Lepus granatensis* world distribution.** The species is native to the Iberian Peninsula and its geographic range includes Portugal and nearly the entire extent of Spain (yellow colour). *L. granatensis* was introduced in the South of France and Balearic islands (purple colour). This map was based on the IUCN distribution map and edited according to (Acevedo et al., 2012).

1.2.2 Genetic diversity

There are three Iberian hare subspecies: i) *L. g. granatensis* (Rosenhauer, 1856) which occupies most of the distribution area; ii) *L. g. solisi* (Palacios, 1992), restricted to Mallorca island and presenting typical insular features, namely shorter posterior extremities, and; iii) *L. g. gallaecius* (Miller, 1907), found in the northwest of the Iberian Peninsula and in Galicia and Asturias (Spain), and distinguished from the nominal by a darker fur colour and longer posterior extremities (reviewed in (Duarte, 2000)).

1.2.3 Morphologic aspects

The Iberian hare *L. granatensis* is larger than the European wild rabbit but smaller than the *L. europaeus* and *L. castroviejoi* (reviewed in (Duarte, 2000)). Morphologically, the *L. granatensis* presents a grizzled yellow-brown fur colour, except in the ventral body region area, where it is

white. Also, the Iberian hare has a black and white fur and the ears are longer with black ear tips (reviewed in (Duarte, 2000)).

L. granatensis is distinguished from the other hare species by a typical white spot in the superior, distal portion of its extremities, absent in *L. europaeus* and *L. castroviejoi* (Duarte, 2000). Also, the white-greyish facial list typical of the *L. castroviejoi* is absent in *L. granatensis*. The white fur colour in the ventral body region area is extensive, creating a clear contrast between the dorsal and ventral body regions, in contrast with *L. castroviejoi*, where this ventral white spot is less extended, and *L. europaeus,* where it is almost absent (reviewed in (Duarte, 2000)).

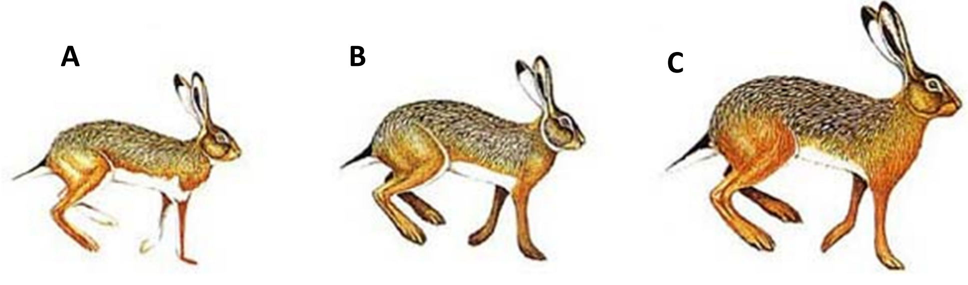


Figure 7. The three hare species found in the Iberian Peninsula. (A)– *Lepus granatensis*; **(B)**- *Lepus castroviejoi*; **(C)**- *Lepus europaeus* (adapted from (Ballesteros, 1998)).

L. granatensis body longitude ranges from 44.4 cm to 47.0 cm. There is sexual dimorphism as females are larger than males. The head-body longitude ranges from 51 cm (in males) to 52 cm (in females) and the maximum body weight varies from 2.95 Kg (in males) to 3.30 Kg (in females) (Purroy, 2011).

1.2.4. Habitat and diet

The *L. granatensis* occupies a wide variety of habitats (Alves et al., 2003), occurring in dry areas as well as in coastal dunes and in the wet mountainous forests (Purroy, 2011). Besides occurring in open fields, the greater species densities occur in intensive agricultural areas (Calzada and Martínez, 1994)(López et al., 1996) such as olive tree and sunflower fields and vineyards (Duarte, 2000). Hares usually feed at night and their diet includes herbaceous plants, mostly leguminous and grasses, but also seeds and fruits, mainly by the end of the summer and autumn, as well as

short bushes. As they prefer springs, agricultural lands with permanent grass renovation are the most favourable habitats for hares (Duarte, 2000).

Hares are solitaire, as they do not have a social organization nor inhabit in borrows (Duarte, 2000). However, they can gather in groups following complex age-dependent patterns, mostly during the feeding time, hence reducing predation risk and increasing feeding efficiency (Marboutin and Péroux, 1999).

1.2.5. Population dynamics

The Iberian hare reproductive strategy, of continuous procreation (Alves and Rocha, 2003), is concordant with smaller litters and longer breeding seasons (Fernandez et al., 2008). The onset of sexual activity is not season dependent but rather on the size of the animals (>1.75-2 Kg) (Purroy, 2011).

In their study on the reproductive biology of *L. granatensis* in southern Iberia, Farfán et al. (2004) found that sexually active males and females appeared in every month except August. Reproductive activity was as its maximum from February to June and reproductively hyperactive females (simultaneously pregnant and nursing) appeared in every month except in January, with a first peak in March and a second lower peak in May–June. Births occurred every month and were more frequent between March and July. Litter size varied from one to seven leverets, but the most frequently one or two. The mean annual litter size was 2.08 and the average number of litters per productive female per year was estimated to be 3.48. The total annual production of young per adult female was estimated to be 7.21.

Hares' abundance is directly related to females breeding success as well as to juvenile survival rates (Marboutin et al., 2003), both directly depend on habitat suitability (Duarte, 2000). The highest juvenile mortality is observed after the maximum reproductive intensity period. Nearly 60% of the young die, corresponding to an increase of 40% in the population numbers (Duarte, 2000). Prenatal mortality was estimated to be between 18% to 21% (Alves et al., 2002)(Fernandez et al., 2008). The minimum annual survival rate of young was 27.91% (Farfán et al., 2004).

The Iberian hare population dynamics is greatly affected by food availability (Carro et al., 1999). Habitat loss, mostly due to the abandonment of agricultural land, can influence negatively the Iberian hare population dynamics. Habitat management is considered the best strategy to recover species numbers (Duarte, 2000).

1.2.6. Importance in the Iberian Peninsula

The Iberian hare plays an important socio-ecological role in the Iberian Peninsula. However, data on the *L. granatensis* ecology is scarce in the international literature, since most information has been almost entirely published in regional journals, books or reports, with limited access (Acevedo et al., 2012).

1.2.6.1 Relevance as prey

Due to the decrease in *O. cuniculus* numbers, hares also play an important role as prey for a large number of predators, including the *Vulnerable* Imperial eagle (*Aquila adalberti*) (Gortazar et al., 2007)(Acevedo et al., 2012).

1.2.6.2. Relevance as a game species

Hares are important game species throughout Europe (Gortazar et al., 2007). The *L. europaeus* is the most important small game species in the old continent, with more than 5, 000, 000 individuals harvested annually (Flux and Angermann, 1990)(Gortazar et al., 2007). The *L. granatensis* has also been identified as an important game species in the Iberian Peninsula (Gortazar et al., 2007), where the species is intensively hunted throughout its range (Duarte, 2000). In Spain, more Acevedo et al., (2012) refers that more than 900, 000 hares were harvested annually.

Currently in Portugal, the Iberian hare can be hunted during the autumn–winter season, which begins at September the 1st and terminates at the end of February (Instituto da Conservação da Natureza e das Florestas, IP, (ICNF)).

In the five years period that ranged from 2011 to 2015 (Table 2), a decrease in the number of Iberian hares hunted in Portugal was registered. However, the negative trend indicated by these data may reflect some bias since the period analysed corresponds to RHDV2 introduction in Portugal, when several game management strategies were applied to stabilize the *O. cuniculus* populations downsizing. Those measures included predator control but also reducing hunting pressure, the latter also influencing the harvest of the Iberian hare.

1.2.7. Hares as putative sentinels for tularaemia in the Iberian Peninsula

Wildlife, as well as pets and farm animals, can be used as clinical sentinels in surveillance systems, each serving distinct roles in disease detection and risk assessment (Gubernot et al., 2008). There is

scientific evidence that sentinel surveillance is predictive of human risk (Gubernot et al., 2008). Animals share environments with humans, respond in analogous ways to many toxic or infectious agents, and clinical signs may manifest in animals prior to humans (Gubernot et al., 2008).

Hares are considered reservoirs of tularaemia, an important zoonotic bacterial disease only recently detected on the Iberian Peninsula (Quijada et al., 2002)(Gortazar et al., 2007)(Lopes de Carvalho et al., 2007)(Lopes de Carvalho et al., 2016). Tularaemia incubation period is slightly shorter in animals and often fatal in wild animals, therefore providing useful means for its detection.

L. europaeus is considered a very good indicator of *Francisella tularensis* presence and activity in natural *foci*, and has been used routinely for the surveillance of this zoonosis (Bandouchova et al., 2011). It is possible to plot a prediction map of tularaemia geographic distribution using data on European brown hares (Pikula et al., 2004)(Bandouchova et al., 2011). The Iberian hare is also susceptible to tularaemia (Duarte, 2000), highlighting the need of monitoring this species.

Table 10. Iberian hare (*L. granatensis*) specimens hunted in Portugal mainland between the years 2005 and 2016, during the cinegetic period (from the September 1st to the end of February). Data was provided by Dr. Gonçalo Lopes from the Instituto da Conservação da Natureza e das Florestas, IP (ICNF). To compare with the homologous data referring to wild rabbits (*O. cuniculus*) hunted in Portugal during the same period Cf Table 1, page 18.

Species		Cinegetic period										
		2005/6	2006/7	2007/8	2008/9	2009/10	2010/11	2011/12	2012/13	2013/14	2014/15	2015/16
Iberian hare (*L. granatensis*)	Nº of hunted specimens	81,371	87,677	105,301	118,049	107,028	104,053	92,401	72,196	37,268	53,768	37,390

1.3. Rabbit haemorrhagic disease (RHD)

Rabbit haemorrhagic disease (RHD) is a highly contagious and fatal disease caused by the RHD virus (RHDV), which affects both wild and domestic rabbits. Until 2010, RHD was attributed to the classical RHDV strains distributed within six well-defined genogroups (G1 to G6, the latter also designated RHDVa) (Le Gall et al., 1998)(Nowotny et al., 1997)(Le Gall-Reculé et al., 2003). However, in late 2010 a new virus emerged in France (Le Gall-Reculé et al., 2011a), representing a distinct lineage of pathogenic rabbit lagoviruses, soon designated RHDV2 (Le Gall-Reculé et al., 2013) or RHDVb (Dalton et al., 2012).

Both RHDV and RHDV2 belong to genus *Lagovirus* of the *Caliciviridae* family (Capucci et al., 1996a)(Le Gall et al., 1998)(Le Gall-Reculé et al., 2013) along with the European brown hare syndrome virus (EBHSV) (Capucci et al., 1996a) and the non-pathogenic lagoviruses (NP-LV), in accordance with the nomenclature suggested by (Le Gall-Reculé et al., 2011b). The general aspects of the *Caliciviridae* family are addressed in Chapter I (**Study 1**).

1.3.1. Historic perspective on the emergence of RHDV and RHDV2

RHD was described for the first time in the Wuxi City, Jiangsu Province of China in 1984 about 100 Km from Shanghai (Liu et al., 1984), in Angora rabbits imported from the German Democratic Republic (Liu et al., 1984), and was associated with high morbidity and mortality rates (Liu et al., 1984)(Xu, 1991). Soon after its emergence, this disease was designated rabbit "Haemorrhagic septicaemia" or "Infectious necrotic hepatitis" (Marcato et al., 1988)(Mitro and Krauss, 1993).

The disease aetiological agent was found to be a RNA virus later designated rabbit haemorrhagic disease virus (RHDV) (Liu et al., 1984)(Xu, 1991). After its first report in the mid 80's, RHDV rapidly spread to an area of approximately 50,000 km^2, killing 140 million domestic rabbits in China in less than a year (Liu et al., 1984)(Xu, 1991). From China, the virus quickly expanded, either in infected rabbits or rabbit products, to other Asian countries, namely to Korea (Park et al., 1987), and latter to Europe, where it was first reported in 1986 in Italy (Cancellotti and Renzi, 1991).

In Europe, RHDV promptly disseminated from domestic to wild rabbits and soon was well established (Cooke, 2002). The first RHD cases in wild rabbits were reported in Spain in 1988 (Argüello-Villares et al., 1988). In Portugal the disease was first described in Madeira Island also in 1988 and in the next years in several islands of the Azorean archipelago (in the islands of Faial in 1988, São Jorge in 1989 and Santa Maria in 1990 (reviewed in (Duarte et al., 2014)). The disease

reached Portugal mainland in 1989 (reviewed in (Duarte et al., 2014)) and was responsible for a dramatic reduction in the Iberian wild rabbit populations (Delibes-Mateos et al., 2007). The disease was also described in France in 1988 and by 1989 the virus was widespread throughout Europe (Morisse et al., 1991)(Le Gall, et al., 1988).

RHDV was reported in 1988 in Mexico, from where it was successfully eradicated since *O. cuniculus* is not a native species (Gregg et al., 1991), as well as in Russia, Middle East and Africa (Morisse et al., 1991). The disease reached India in 1989 (Morisse et al., 1991) and North America in 2000 (McIntosh et al., 2007). As the virus spread worldwide, naturally occurring RHD outbreaks were reported in geographically distant regions, such as Cuba (Farnós et al., 2007) and Uruguay (McIntosh et al., 2007).

In marked contrast with other countries that faced the disease with concern, Australia used RHDV as a viral bio-control agent for rabbits, considered an agricultural pest and a major threat to the endemic flora and fauna wildlife (Jahnke et al., 2010). Due to its exceptional virulence, high transmissibility, and narrow host range, the RHDV Czech reference strain (CAPM-V351) was released in 1991 in the Wardang Island in Spencer Gulf, South Australia, after the approval of the Australian authorities (Eden et al., 2015). However, regardless the rigorous quarantine measures, the virus reached the mainland in 1995 possibly transported by insects or air currents (Kovaliski, 1998). In less than two years (the initial spread was estimated to be 50 Km per week) it became established across southern Australia, causing a reduction of more than 95% of the wild rabbit populations in some areas, particularly in the more arid regions (Abrantes et al., 2012). In New Zealand, despite the rabbit is also considered a plague, the government decided not to use the virus as biological weapon. Yet, RHDV was later illegally introduced by landholders (O 'hara, 2006).

In brief, RHDV became endemic in areas where the European rabbit wild populations were historically present, while the occurrence of RHDV as an epidemic or rare outbreaks seemed to occur in countries where the rabbit was mainly present as a domestic or industrial animal and related with rabbit colony number and density (Abrantes et al., 2012).

RHDV2 is a new pathogenic lagovirus, which differs from RHDV in terms of phylogenetic position, antigenic profile and pathogenicity (Le Gall-Reculé et al., 2013). The first RHDV2 reported cases took place in April and May 2010, in a rabbitry in western France and in a wild rabbit, respectively. Until December of that year, the estimated mortalities rates caused by RHDV2 in wild rabbit populations ranged from 80% to 90% (Le Gall-Reculé et al., 2011a) and RHDV2 was responsible for

75% (25/34) of the RHD epizootics recorded in 2010, mainly in north-western France (Le Gall-Reculé et al., 2013). As early as February 2011, RHDV2 had already reached the south of France, demonstrating the large-scale spread of the virus (Le Gall-Reculé et al., 2013). RHDV2 was responsible for 73% (29/40) and for 95% (41/439) of the epizootics recorded between January and June and between July and December 2011, respectively (Le Gall-Reculé et al., 2013).

After crossing the Alps and the Pyrenees geographic barriers, favoured by the lack of cross-protection conferred by classic strains induced immunity (Le Gall-Reculé et al., 2013), RHDV2 quickly disseminated to other European countries, including Spain (Dalton et al., 2012) and Italy (Le Gall-Reculé et al., 2013), were it was first detected in 2011. The virus reached Portugal mainland in late 2012 (Abrantes et al., 2013). It was reported in wild rabbits collected near the Spanish border (Abrantes et al., 2013) from where it rapidly dispersed to the centre and south of the country in a very short period of time (Lopes et al., 2015a). Germany (information on the FLI, 10|21|2013), England and Wales (Westcott et al., 2014) and Scotland (Baily et al., 2014) reported RHDV2 in 2013. By January 2015, RHDV2 had already extended to several islands of the Azorean archipelago (Duarte et al., 2015a). Also in 2015, the virus was reported in Australia (Hall et al., 2015), Finland (http://www.oie.int/wahis_2/public/wahid.php/Countryinformation/Countryreports) and Tenerife Island (Martin-Alonso et al., 2016). Tunisia, the Scandinavian countries of Norway (OIE Technical disease cards. 2016), and Sweden (Neimanis et al., 2017), the Ivory Coast (http://outbreakwatch.blogspot.pt/2016/09/proah-rabbit-hemorrhagic-disease-cote.html) and Canada (http://outbreakwatch.blogspot.pt/2016/08/proahedr-rabbit-hemorrhagic-disease_26.html) reported the disease in 2016. Recently, by the end of 2016, the virus was also detected by our team in the Madeira archipelago (Carvalho et al., 2017c).

1.3.2. Aetiological agent

1.3.2.1. Virion structure

Classic RHDV is the prototype virus of the genus *Lagovirus*. However, it was not until early 1990s that the virus was finally assessed in as a member of the *Caliciviridae* family, since the first attempts to classify RHDV were erratic due to its non-cultivable nature (reviewed in (Abrantes et al., 2012)). RHDV is a non-enveloped small sized virus, which outer diameter varies between 32-35 nm (with a range of 28-42 nm) with a positive-sense, single-strand RNA genome of approximately 7.4 kb (7437 nucleotides long) (Valícek et al., 1990)(Capucci et al., 1991)(Wirblich et al., 1996)(Meyers et al., 2000)(Dalton et al., 2015) and a characteristic morphology defined by cup-shaped depressions

(**Figure 8A and B)** (Valícek et al., 1990)(Capucci et al., 1991)(Sibilia et al., 1995). A high percentage of viral particles present an electron-dense core with an approximately 23-25 nm diameter, delineated by a rim from which radiate ten short (5 to 6 nm long) peripherical projections, regularly distributed (Capucci et al., 1991)(Sibilia et al., 1995).

Besides the genomic RNA (gRNA), RHDV virions also contain an abundant subgenomic mRNA (sgRNA) with 2.2 kb, which is collinear with the 3′ end of the genomic RNA (Meyers et al., 1991a)(Wirblich et al., 1996)(Meyers et al., 2000). Both the genomic and the subgenomic RNAs are packaged in non-enveloped icosahedral capsids that consist of the major structural protein VP60 (Wirblich et al., 1996).

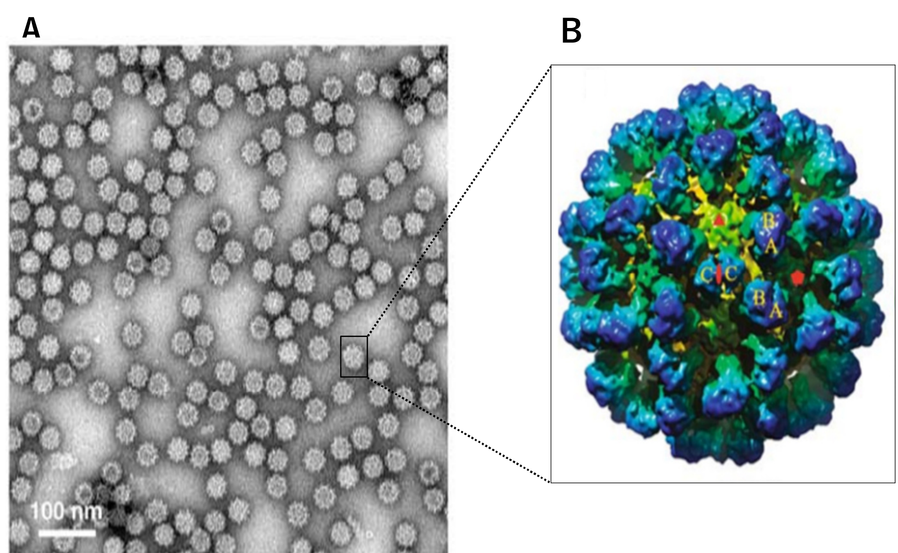


Figure 8. Rabbit haemorrhagic disease virus (RHDV). (A)- Micrograph of purified, negatively stained RHDV (bar=100 nm) (adapted from (Wang et al., 2013); **(B)**- Reconstructed intact RHDV virion with diameter ~41nm (adapted from (Hu et al., 2010)).

1.3.2.1.1. VP60 (VP1) capsid protein

The viral capsid forms a layer that protects the RNA molecule and comprises 180 copies of a single capsid protein (Bárcena et al., 2004)(Luque et al., 2012). Virions consist of 90 capsid protein protruding arch-like dimeric capsomers which surround 32 large hollows or cup-shaped depressions (only visible in the virus purified form) arranged in a T=3 icosahedral symmetry (Valícek et al., 1990)(Capucci et al., 1991)(Thouvenin et al., 1997)(Bárcena et al., 2004).

Each capsid protein monomer consist of shell (S) domain, involved in forming the icosahedral shell which protects the viral RNA, with an arm at the N-terminal region (the N-terminal arm or NTA)

facing the inner surface of the capsid shell and connected by a hinge to a flexible protruding (P) domain (**Figure 9**) (Bárcena et al., 2004)(Luque et al., 2012)(Bárcena et al., 2015). The (P) domain encompasses the C-terminal region and forms the prominent protrusion emanating from the shell (Bárcena et al., 2004). While the VP60 N-terminal region is buried within the particle, the C-terminal region is exposed on the surface (Bárcena et al., 2004). The (P) domain can be further subdivided into the subdomains P1 (the stem or leg of the protrusion) and P2 (top of the protrusion) (**Figure 10 B and C**) (Hu et al., 2010)(Bárcena et al., 2015). While the S domain (**Figure 10A**) is well conserved, the P domain contains determinants for virus-host receptor interactions and antigenic diversity (Bárcena et al., 2004)(Hu et al., 2010)(Luque et al., 2012)(Bárcena et al., 2015). The P1 sub-domain is only moderately conserved and the P2 sub-domain, located at the outermost surface region of the viral capsid protein, is highly variable (Hu et al., 2010)(Bárcena et al., 2015), displaying the greatest genetic and antigenic variation (Bárcena et al., 2015).

RHDV VP60 domains

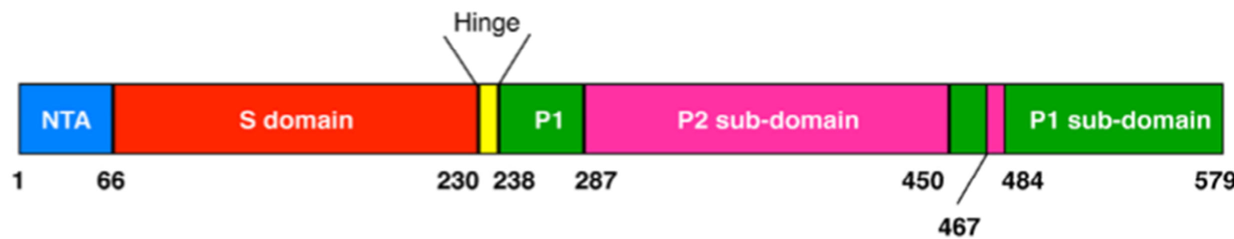


Figure 9. Domain organization of RHDV VP60. The Shell (S) domain encompasses amino acids (aa) 66 to 230. The Protruding (P) domain comprehends aa 238 to 579 The P domain is further subdivided in the P1-subdomain, encompassing aa 238 to 287, 450 to 467 and 484 to 579. The P2-subdomain comprehends aa 287 to 450 and 467 to 484 (adapted from (Wang et al., 2013)).

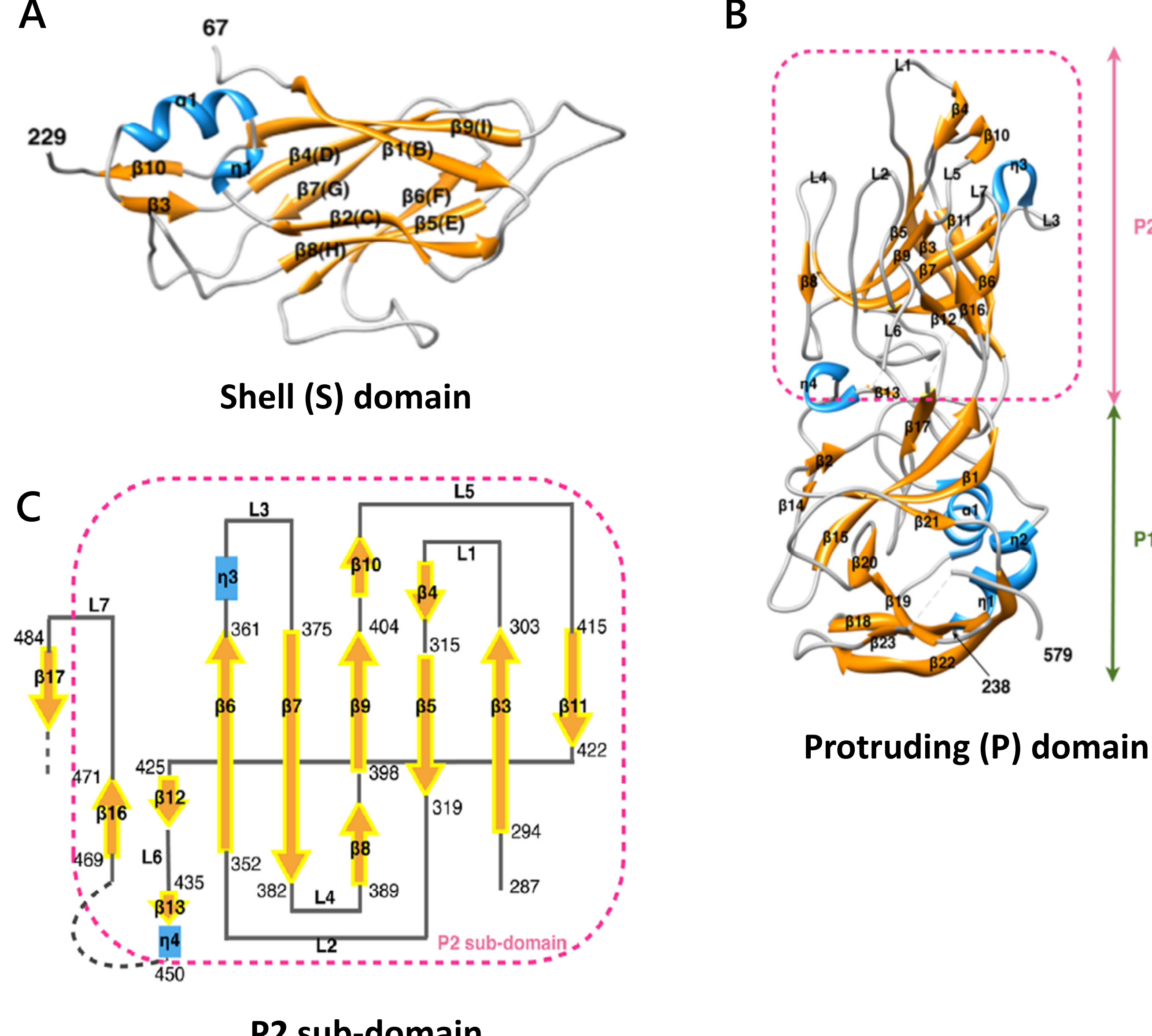


Figure 10. Ribbon representation of the crystal structure of the RHDV VP60 S and P domains and topology diagram of P2 subdomain. (A)- S (Shell) domain; secondary structures are coloured blue for helices, gold for β-strands, and grey for loops and are labelled sequentially. **(B)-** P (Protruding) domain; P1 (green) and P2 (pink) sub-domains are indicated and coloured according to their secondary structure elements (blue for helices, gold for β-strands, and grey for loops) and labelled sequentially. **(C)-** Topology diagram of the VP60 P2 sub-domain (labels and residue numbers correspond to those shown in panel **(B)**); (adapted from (Wang et al., 2013)).

RHDV capsomers have a hat-shaped prominence at the P2 region, indicating a specific antigen structure and antibody binding sites (Hu et al., 2010). Each dimeric capsomer presents two monoclonal antibodies (MAb) binding sites (Thouvenin et al., 1997). The greater amino acidic variability found in P2 subdomain is due, at least in part, to the selection pressure imposed by the host antibodies that recognise and target regions located in this subdomain (Martínez-Torrecuadrada et al., 1998)(Bárcena et al., 2004). In order to avoid this recognition, these regions tend to evolve faster having higher genetic variability and antigenic variation (Esteves et al., 2008)(Kinnear and Linde, 2010). The RHDV antigenic variant G6 or RHDVa virus-*like* particles (VLP) pseudo-atomic model determined by Wang et al., (2013) enabled the identification of seven regions of sequence variation on the P domain, which are addressed in a more comprehensive manner in Chapter V "RHDV and RHDV2 genetic relations".

The specific packing among the 90 RHDV capsomers and the different interactions among adjacent capsomers indicates that the P domain, especially the P2 subdomain, has different fold and conformation when compared with other caliciviruses (Hu et al., 2010). Capsids are dynamic structures whose components have transient conformations in relation to specific functions in the viral cycle (Luque et al., 2012). In capsids with triangulation numbers T = 3 (**Figure 11A** and **B**), the icosahedral asymmetric unit consists of three quasi-equivalent subunits, as the single capsid protein must be able to adopt three slightly different conformations (A, B, and C), because they must adapt to three quasi-equivalent but different structural environments in the shell (Harrison, 2001). In accordance, RHDV VP60 (**Figure 11 A** and **B**) is able to switch among quasi-equivalent conformational states by a mechanism involving the N-terminal region of a subset of subunits (Bárcena et al., 2004). Also, the C-terminal (whose residues mostly face the hollows of the cup-shaped depressions) is also involved in inter-dimeric contacts (Bárcena et al., 2004). Deletions of C-terminal amino acid residues may result in misfolding or altered conformation of the remaining peptide chain, impeding the VLP assembly (Bárcena et al., 2004), (**Figure 11C**).

RHDV VP60 three-dimensional structures

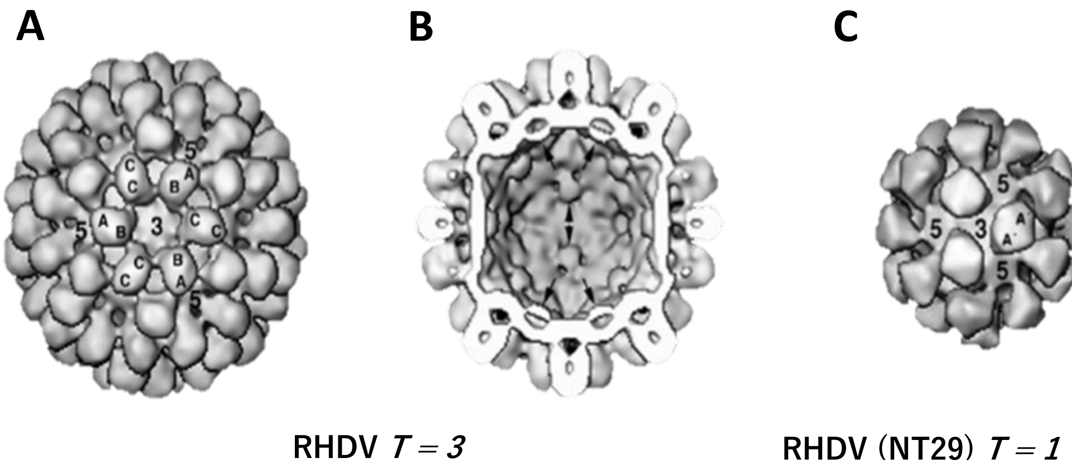


Figure 11. RHDV VP60 three-dimensional structures. (A)- Surface-shaded representation of the outer surface of the VP60 capsid viewed along a 3-fold axis of icosahedral symmetry. The positions of the three conformers of VP60 (A, B, and C) are indicated. **(B)**- A model of the VP60 capsid with the front half of the protein shell removed viewed along a 2-fold axis. Six lid-shaped structures located on the inner surface at the 3-fold axis are indicated by arrows. **(C)**- Surface-shaded representation of the outer surface of small NT29 capsids viewed along a 3-fold axis of icosahedral symmetry. T = 1 dimers are shown with the opposite handedness with respect to T = 3 dimers. Two monomers of NT29 protein are indicated (A′) (adapted from (Bárcena et al., 2004)).

The capsid protein VP60 is the main target of the host immune defence against RHDV, and plays an important role in virus diagnosis and vaccine design (Esteves et al., 2008).

X-ray crystallography (Adams et al., 2010) has facilitated the construction of accurate models for the VP60 domains of RHDV (Wang et al., 2013) and RHDV2 (Leuthold et al., 2015), allowing the comparison of those structures between both viruses (Bárcena et al., 2015). The P domains sequence identity between RHDVa (RHDV G6) and RHDV2 found by Leuthold et al., (2015) was 84% and superposition of RHDV2 and RHDVa P domains showed a similar overall structure. However, the RHDV2 P1 subdomain helixes were slightly shifted and a number of P2 subdomain loops were oriented differently. Also, the three extended loop regions located at the outer surface of the P2 subdomain contained randomly distributed amino acid variations. The implication of RHDV2 P

domain loop different conformation will be addressed in more detail in section "1.3.2.3.1. Histo-blood group antigens (HBGAs) attachment factors".

1.3.2.1.2. VP10 (VP2)

VP10 is proposed to be a basic minor structural protein with 12,7 KDa (Liu et al., 2008)(Chen et al., 2009) and a component of RHDV virions (Wirblich et al., 1996)(König et al., 1998)(Meyers et al., 2000) where it is present in small amounts (Esteves et al., 2008). The VP10 is expressed by an unknown mechanism (Meyers et al., 2000) and its expression level is considered to be very low, approximately one-fifth (≈ 20%) of the VP60 expression level (Meyers, 2003).

The VP10 protein is conserved in all caliciviruses, suggesting that it may play an important role in the virus life cycle (Liu et al., 2008)(Chen et al., 2009), but the precise biological function of RHDV VP10 is unclear (Liu et al., 2008). Two main hypotheses have been considered regarding its function. The first, hypothesised that the VP10 protein could play a role in virus-particle assembly by interacting with both VP60 and the viral RNA (or the VPg protein linked to RHDV-RNA), thus mediating specific encapsidation of the virus genome (Sosnovtsev et al., 2005). The second assumed that VP10 could be a regulatory factor related to virus replication, protein expression or virus-induced cell apoptosis (Liu et al., 2008)(Chen et al., 2009).

Regarding the first hypothesis, although studies on other members of the *Caliciviridae* family, such as the feline calicivirus (FCV) (Sosnovtsev et al., 2005), have demonstrated that the VP10 is critical for infectivity, RHDV was shown to retain its infectivity in the absence of VP10 (Liu et al., 2008). In addition, virus-like particles (VLPs) have been produced by expressing only the VP60 protein in insect cells (Laurent et al., 1994). These results suggest that that VP10 is not essential for the production of infectious RHDV virions (Liu et al., 2008), in contrast with previous assumptions (Wirblich et al., 1996)(Glass et al., 2000)(Sosnovtsev and Green, 2000).

Although VP10 may not participate in the production of progeny virions, it may represent an important virulence gene (Liu et al., 2008). *In vitro* and *in vivo* studies showed that RHDV VP10 downregulates the expression of the viral capsid protein VP60 (Chen et al., 2009) and reduces the levels of sgRNA (Parra et al., 1993), the major source of capsid protein assembled into mature virions. The mechanisms by which this occurs are not yet fully understood and, although it seems that the VP60 downregulation occurs at the transcriptional level, the possibility that VP10 may also regulate VP60 protein expression at the translational or even post-translational level cannot be excluded (Chen et al., 2009).

The VP10 ability to regulate capsid protein levels may contribute to effective virus infection (Chen et al., 2009). It seems that VP10 might regulate virus replication, by reducing it (Liu et al., 2008). In addition, VP10 seems to be involved apoptosis induction following infection and in virion release from infected host cells (Liu et al., 2008)(Chen et al., 2009), a determinant process in the development of the RHDV pathogenesis (Alonso et al., 1998)(Jung et al., 2000). Studies in feline calicivirus (FCV) have demonstrated that virus replication and *de novo* synthesis of viral proteins are critical for the induction of apoptosis (Sosnovtsev et al., 2003).

1.3.2.1.3. RHDV Core-like particles (CLP)

A second type of classical RHDV virions was also reported to be obtained sporadically from rabbits' liver with subacute or chronic RHD (Granzow et al., 1996). These viral particles were designated core-like particles (CLP), smooth particles or s-RHDV (Granzow et al., 1996). CLP were found in the liver and spleen but not in the bloodstream (Barbieri et al., 1997)(Hu et al., 2010) and correspond to the assembly of the N-terminal region of the RHDV-VP60 (Laurent et al., 2002) into small (25-27 nm in diameter), and smooth particles which present only one structural protein of 28-30 kDa (**Figure 12**) (Granzow et al., 1996)(Laurent et al., 2002)(Hu et al., 2010). CLP have no haemagglutinating properties, most likely due to the absence of the C-terminus, but present reactivity with sera from RHDV convalescent rabbits and monoclonal antibodies directed towards the N-terminal part of the RHDV capsid protein (Granzow et al., 1996)(Barbieri et al., 1997). CLP seem to be associated with the appearance of specific anti-RHDV IgM (Abrantes et al., 2012) and it was suggested that they might result from the degradation of RHDV-IgM immuno-complexes, formed in large amounts at the beginning of the humoral response (Barbieri et al., 1997)(Hu et al., 2010). Although it was also suggested that CPL could result from a truncated VP60 genome or defective expression rather from proteolytic degradation (Granzow et al., 1996)(Bárcena et al., 2004), more recent date showed that CLP directly derive from intact virions with dissociated protrusion (Hu et al., 2010).

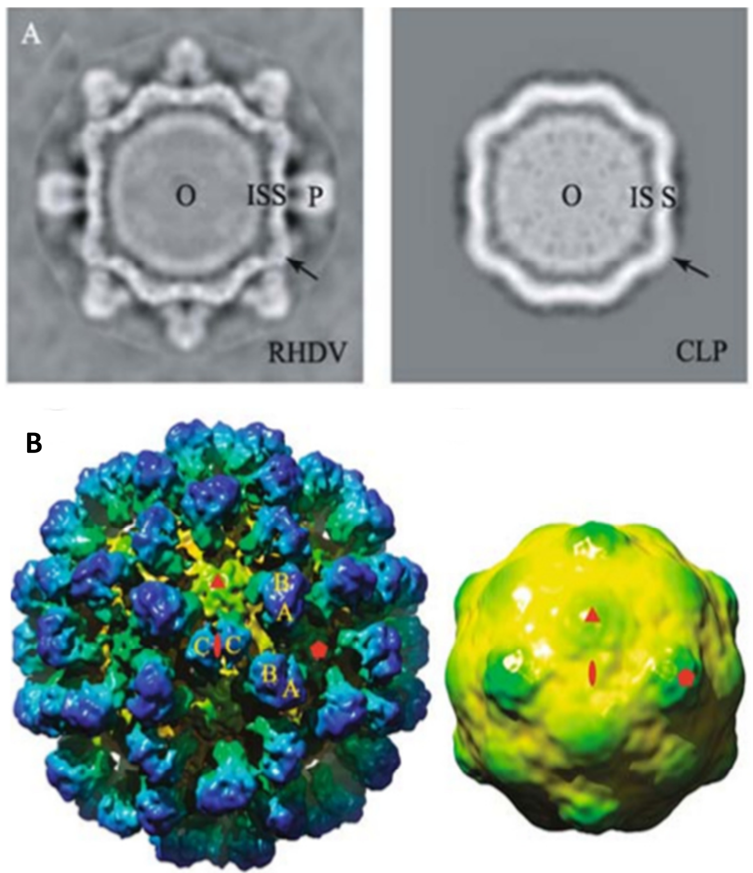


Figure 12. Comparison between intact RHDV virion and CLP. (A)- Central section perpendicular to one of 2-fold axes plotted against the intact RHDV virion and CLP. The RHDV virion contain four regions of densities, from the centre to outside: RNA containing core (O), inner shell (IS), capsid shell (S) and capsid protrusion (P); the arrows indicate the similar features between two structures (adapted from Hu et al., 2010). **(B)**- Isosurface representation of both intact RHDV virion (diameter ~41nm) and CLP (diameter ~32nm) (adapted from (Hu et al., 2010)).

1.3.2.2 RHDV genomic organization

Caliciviruses' genomes are typically organized into either two (*Lagovirus*, *Sapovirus*, *Nebovirus*) or three (*Norovirus*, *Vesivirus*, *Recovirus*) major open reading frames (ORF) (reviewed in (Taube et al., 2010)), although a fourth ORF was identified in murine norovirus (MNV) (Thackray et al., 2007). The RHDV gRNA consists of two slightly overlapping ORFs. ORF1 has 7 kb and comprises nucleotides 10 to 7044 while ORF2 is 351 nucleotides-long, from nucleotide 7025 to 7378, covering nearly 99% of the genome (Meyers et al., 1991b)(Wilblich et al., 1996).

1.3.2.2.1. ORF1

ORF1 encodes a primary translation product of 257 kDa (Meyers et al., 1991b), containing the non-structural genes (grouped in the 5′ region) followed by the 60 kDa capsid protein genes (in the 3′ region), uninterruptedly (Meyers et al., 1991b)(Parra et al., 1993). This is a distinguishing characteristic from other caliciviruses (Capucci et al., 1996). The genome organization of RHDV is given in **Figure 13**.

The 257 kDa polyprotein contains the non-structural and major capsid proteins, which are arranged in the order NH2-p16-p60-p41-p72-VP60-COOH (König et al., 1998)(Meyers et al., 2000). The p60 is further cleaved into p23 and p37 (a helicase) and, following an apparently complex processing system, p41 gives rise to products of 23 and 18 kDa (p23/2 and p18), or alternatively, to polypeptides of 29 and 13 kDa (p29 and p13) (Meyers et al., 2000). The latter product represents VPg (Meyers et al., 2000). Apparently, cleavage of p41 into p29 and p13 (p14) seems to be preferred in infected cells or, alternatively, p18 is rapidly processed to give rise to p13 and a hypothetical product of 5 kDa not detected so far (Meyers et al., 2000). The p72 represents a fusion protein composed of the viral cysteine protease (p15) and the RNA-dependent polymerase (RdRP, p58) in which is cleaved with rather low efficiency (Wirblich et al., 1996)(Meyers et al., 2000)(Thumfart and Meyers, 2002). According to the above described genome organization, cleavage of the ORF 1 polyprotein occurs at eight cleavage sites (Thumfart and Meyers, 2002). Most, if not all, cleavages are performed by a virus-encoded trypsin-like cysteine protease (TCP) showing significant similarity to the 3C proteases of picornaviruses (Wirblich, 1996)(König et al., 1998)(Meyers et al., 2000)(Thumfart and Meyers, 2002). Seven of the eight sites were identified and correspond to the dipeptides located in the positions 143/144 (EG, p16-p23), 367/368 (EG, p23-p37), 718/719 (EG, p37-p41), 993/994 (QG, within p41), 1108/1109 (EG, p13(VPg)-p15(protease), 1251/1252 (ET, p15 (protease)-p58 (polymerase) and 1767/1768 (EG, p58 (polymerase)- VP60) (Martín-Alonso et al., 1996)(Wirlblich et al., 1996)(Meyers et al., 2000). The still unknown site is located within p41 and separates the processing products p23/2 and p18 (Thumfart and Meyers, 2002). Blocking of EG 1108/1109 prevents the generation of p41, p13 (VPg), p72 (protease/ RdRp), and p15 (protease)(Meyers et al., 2000) while blocking cleavage at site 718/719 had a major effect on processing of p41 in general (Meyers et al., 2000).

The sgRNA also contains the entire VP60 gene and, apparently, is responsible for the synthesis of most or all of the VP60 assembled into mature virions (Parra et al., 1993)(Sibilia et al., 1995). In fact, as the assembly of virus like particles (VPLs) occurs irrespectively of the pathway followed for the

VP60 synthesis, it is possible that capsid synthesis follows two distinct pathways, one through processing of the polyprotein precursor translated from the gRNA and the second by translation of the sgRNA (Meyers et al., 1991a)(Parra et al., 1993)(Sibilia et al., 1995).

The helicase and the RNA-dependent RNA-polymerase (RdRp, which catalysis VPg uridylation) are involved in the replication of the viral RNA. The protease is responsible for the proteolytic processing of the large polyprotein. However, the function of the non-structural proteins p16, p23 and p29 is still to be determined (Abrantes et al., 2012).

It is noteworthy that, in other caliciviruses the capsid protein is encoded by a separate ORF (Meyers et al., 1991b)(Parra et al., 1993).

1.3.2.2.2. ORF2

ORF2 is located at the 3' end of the genome and encodes VP10, a putative minor structural protein of 117 amino acids, also encoded by the sgRNA (Sibilia et al., 1995).

The gRNAs express products needed during intermediate and late stages of infection, such as structural or movement proteins (Miller and Koev, 2000). Because replication is required for sgRNA synthesis, the RNA-dependent RNA polymerase (RdRp) is always translated first, directly from genomic RNA of positive-strand RNA viruses (Miller and Koev, 2000). Both the genomic and subgenomic RNAs are polyadenylated and covalently linked through a Tyr-21 residue at the 5' end to a 15-kDa virus-encoded protein, VPg (Meyers et al., 1991a,b)(Sibilia et al., 1995)(Meyers et al., 2000)(Machín et al., 2001), constituted by 115 amino acid residues with a putative role in RHDV genome synthesis initiation (Machín et al., 2001).

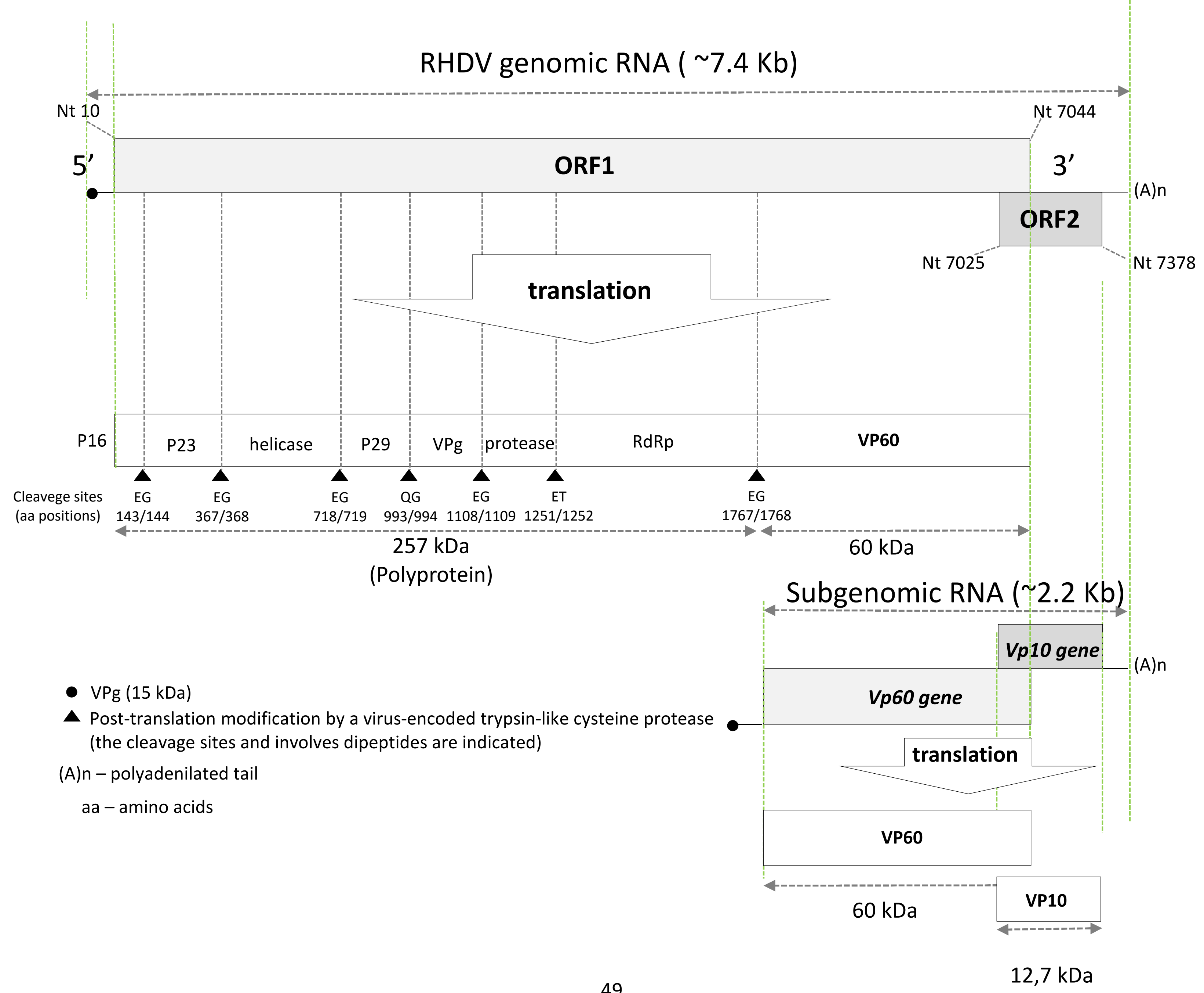


Figure 13. Genome organization of RHDV. Both genomic and subgenomic RNA and open reading frames (ORF) 1 and 2 are represented. The translational products and the 257 kDa polyprotein cleavage sites are represented as well (adapted from (Wilblich et al., 1996)(Martín-Alonso et al., 1998) and (Meyers et al., 2000)).

RHDV2 shares with RHDV the same genomic structure, organized in two potential open reading frames (ORFs) (Dalton et al., 2012). The complete genome sequence of two RHDV2 isolates strain collected on the Iberia (RHDV-N11 from Spain and CBVal16 from Portugal) was recently obtained by Dalton et al. (2015). The RHDV-N11 strain genome is 7447 nucleotides (nt) in length. Both isolates showed an identical 5′ untranslated region (UTR) of 9 nt. The 3′ UTR of RHDV-N11 is 69 nt long, larger than UTRs of classic RHDV or RHDVa strains (both with 59 nt). The 3′ 22-nt sequence of isolate CBVal16 was not determined; however, there was a 4-nt deletion in the remaining 30 UTR region with regards to RHDV-N11. In RHDV2 strains, ORF 1 encodes a polyprotein with 2344 amino acid residues, which includes the predicted non-structural proteins NS1 (2A, 143 aa), NS2 (2B, 196 aa), NS3 (2C NTPase, 379 aa), NS4 (3A, 275 aa), NS5 (3B, 115 aa), NS6 (3C protease, 143 aa) and NS7 (3D polymerase, 516 aa), and the structural protein VP60 (577 aa and 579 aa when expressed from sgRNA). ORF2, which partially overlaps with the terminal six amino acids of ORF1, encodes the minor structural protein VP10 with 117 aa. The authentic ATG for VP10 has yet to be determined, and there are four in-frame ATGs in the region surrounding the overlap; ATG at nucleotide position 7025–7027 is that which best maps to RHDVAst89 (Dalton et al., 2015).
The potential cleavage sites, conserved in relation to the RHDV Ast89, are E143/G144 (NS1); E338/G339 (NS2), E718/G719 (NS3), Q993/G994 (NS4), E1108/G1109 (NS5), E1251/T1252 (NS6) and E1767/G1768 (NS7). There are divergences in the literature with regard to the C-terminal NS2 processing site, indicated both in amino acids 338/339 and 367/368 (Meyers et al., 2000)(Bull and White, 2010)(Sosnovtsev, 2010). In the RHDV2 sequences, the potential cleavage site E338/ G339 is conserved with respect to RHDVAst89, while the potential cleavage site E367/D368 in classic RHDV sequences is E367/E368 in the RHDV2 sequences. The RHDV2 exact polyprotein-processing sites are yet to be determined experimentally (Dalton et al., 2015).
The sgRNA transcriptional starting site for classic RHDV is at the nucleotide position 5296 in the genome. This region, including the subgenomic promoter region (Morales et al., 2004)(Simmonds et al., 2008) and the 5′ UTR of the subgenomic transcript, is highly conserved (98.2% identity, nt 5266–5322) between the RHDV2 and classic RHDV (RHDVAst89; accession number Z49271) (Dalton et al., 2015).

1.3.2.3. Virus Life Cycle

The hepatocytes cytoplasm is considered the major site of RHDV replication (reviewed in (Abrantes et al., 2012)). However, the epithelial cells of the upper respiratory and digestive tracts, the most probable virus entry doors (Ruvoën-Clouet et al., 2000)(Guillon et al., 2009), were also suggested as primary sites of viral replication (Ruvoën-Clouet et al., 2000).

After RHDV attachment to the cell surface, viral internalisation, by an unknown mechanism, and desencapsidation occurs (Abrantes et al., 2012). The viral genome is released into the cell cytoplasm where the proteins translation is initiated (Abrantes et al., 2012). Translation initiation is a key point for successful gene expression, as well as a potential site for its regulation, and viruses are dependent on the host cell biochemistry for this process (Meyers et al., 2003). The mechanism used by most viral and cellular mRNAs for initiating translation is 5′-end and 7- methylguanosine (m^7G) cap-dependent (Daughenbaugh et al., 2003). A complex set of protein-protein and RNA-protein interactions that begin with binding of eIF4F initiation factor to the 5′ terminal m^7G cap structure on the mRNA, initiate translation (Daughenbaugh 2003). As caliciviruses lack m^7G cap structures (Daughenbaugh 2003), the viral genome linked protein VPg was shown to play a crucial role in the translation initiation for these viruses (Meyers et al., 2003)(Daughenbaugh 2003). VPg interacts with the translation initiation factors eIF4E and/or eIF3 and possibly serves as a cap substitute or analogue (Goodfellow et al., 2005)(Daughenbaugh et al., 2003)(Meyers, 2007). The ORF1 encoded polyprotein precursor translation can then occur at the initiation codon AUG (Abrantes et al., 2012). Translation of ORF2 encoded VP10 starts by an unusual mechanism of re-initiation after termination of translation of the preceding major capsid protein VP60 (Meyers et al., 2003)(Meyers, 2007). The ORF2 translated region starts with codon AUG2 and is dependent on translation of the preceding sequence and the presence of the last 84 nucleotides of ORF1 (Meyers et al., 2003)(Meyers, 2007). The name TURBS ("termination upstream ribosomal binding site") was proposed by Meyer et al. (2003) for this RNA element, located upstream the start/ stop site, which should ensure the prolonged contact of post-termination ribosomes and RNA to allow the binding of the necessary factors for a new translation cycle. Two essential motifs for VP10 expression were recognized within TURBS (Meyers et al., 2003)(Meyers, 2007), motifs 1 and 2, respectively. Motif 1 is highly conserved among caliciviruses and complementary with a short 18S rRNA sequence, which suggests an interaction between the viral RNA and the ribosomal 18S rRNA. Motif 2 is thought to be implicated in the correct ribosome positioning at the translational start site (Meyers, 2007).

Despite AUG2 presence importance for the VP10 yield it can be replaced by non AUG initiation codons (Meyers et al., 2003). The standard ribosome scanning process used for cellular mRNAs cannot be used, as the ORF2 AUG2 represents AUG number 28 in the viral sgRNA and is surrounded by a sequence representing a weak context for translation initiation (CUUAUGU) (Meyers, 2003).

Post-translational proteolytic processing by the viral gRNA encoded protease cleaves the ORF1 polyprotein precursor into the RHDV capsid protein VP60 and mature non-structural proteins (Abrantes et al., 2012). The latter, a helicase and RdRp, form a replication complex and synthesise a complementary negative-sense RNA from the gRNA, used as a template for the gRNA and sgRNA synthesis (Abrantes et al., 2012). The RNA product can be translated again or packaged into viral particles that will be released from the infected cell (Abrantes et al., 2012). Although the mechanisms used by RHDV for egress are not well clarified, VP10 is able to induce hepatocytes' apoptosis and virion release and dissemination (Jung et al., 2000)(Liu et al., 2008). A representation of RHDV lifecycle is given in **Figure 14**

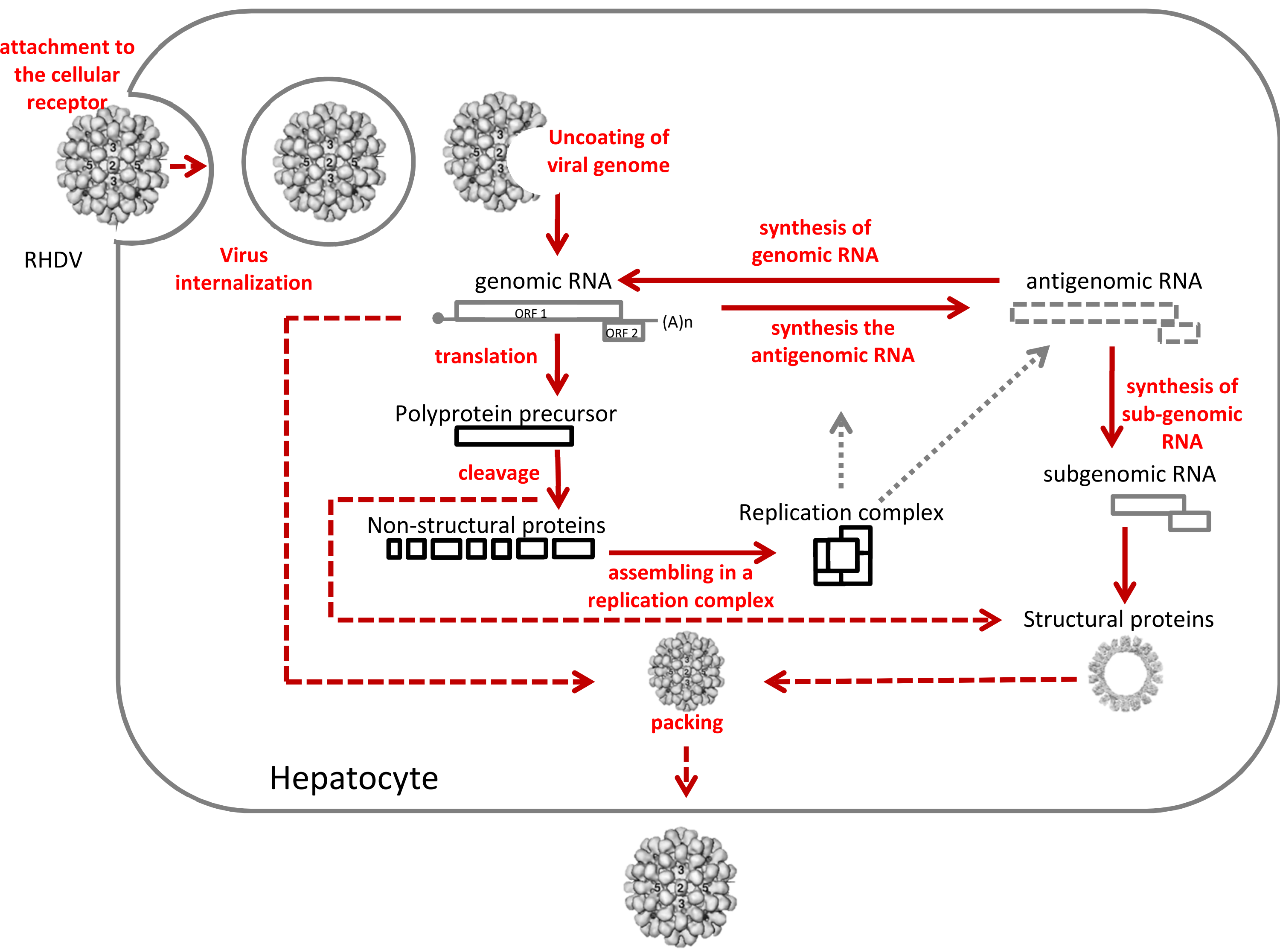


Figure 14. Caliciviruses lifecycle. After attachment to the cellular receptor, the virion is internalised into the cell. Uncoating of the viral genome is followed by translation of the polyprotein precursor and co-translational processing releasing the non-structural proteins. These proteins assemble in a replication complex that synthesises the antigenomic RNA, used as a template for the genomic RNA synthesis. The newly synthesized genomic RNA is translated as a polyprotein precursor or is used for packaging in the assembled viral protein core. The antigenomic RNA is also the template for synthesis of subgenomic RNA. The subgenomic RNA is translated as structural proteins, VP60 and VP10. In lagoviruses, VP60 is also released from the polyprotein precursor after processing by the viral protease. Assembly of the structural proteins as well as packaging of the genomic RNA occurs, followed by release of the mature virion from the cell (adapted from (Abrantes et al., 2012)).

In the absence of a culture system for RHDV, much of our understanding of this virus relies on the experimental infection of rabbits and on recombinant DNA technology for the production of recombinant RHDV virus-like particles (VLP) in heterologous systems (Sibilia et al., 1995)(Nagesha et al., 1995)(Luque et al. 2012). Cloning of the RHDV genome and expression in the baculovirus system have enabled the study of many aspects of this virus (Laurent et al., 1994)(Nagesha et al., 1995). Indeed, the RHDV capsid protein was successfully expressed in insect cells where it spontaneously assembled to form VLPs, physically and immunologically indistinguishable from the intact wild-type virions despite being devoid of viral RNA (Sibilia et al., 1995)(Nagesha et al., 1995) (Clarke and Lambden, 1997)(Bárcena et al., 2004)(Luque et al., 2012). More recently, a recombinant baculovirus expressing the RHDV2 VP60 protein was also produced in insect cells (Bárcena et al., 2015).

1.3.2.3.1. Histo-blood group antigens (HBGAs) attachment factors

RHDV was the first calicivirus shown to bind to ABH histo-blood group antigens (HBGAs) on rabbit epithelial cells of the upper respiratory and digestive tracts (Ruvoën-Clouet et al., 2000). HBGAs are attachment factors or ligands for RHDV, rather than the main cellular receptor, and facilitate RHDV infection (Nyström et al., 2011).

HBGAs are polymorphic carbohydrate structures representing terminally exposed portions of larger glycans linked to proteins or glycolipids (Nyström et al., 2012). HBGAs are built up by sequential addition of monosaccharide units via glycosyltransferase enzymes with affinity to a specific substrate in a process called glycosylation (Ruvoën-Clouet et al., 2000)(Marionneau et al., 2001). Several genes encode the glycosyltransferase enzymes resulting in ABH, Lewis and secretor polymorphic phenotypes (Marionneau et al., 2001). In rabbits, the α1,2-fucosyltransferase involved in the different HBGAs synthesis is encoded by three functional genes, *Fut*1, *Fut*2 and *Sec*1 (Guillon et al., 2009)(Abrantes et al., 2012). The H antigen is considered the building block to produce of the antigens and its deficiency is denominated the "Bombay phenotype" or "O" (Yunis et al., 1969). Bombay individuals lack ABH epitopes due to inactivating mutations in the gene *Fut*1 (Ruvoën-Clouet et al., 2000)(Taube et al., 2010).

In many vertebrate species HBGAs are mainly expressed on epithelial surfaces (Nyström et al., 2011). Yet, a few primate species, including humans, express them on erythrocytes and vascular endothelial cells (Nyström et al., 2011). This distinctive characteristic of human erythrocytes determines the important ability of RHDV to strongly agglutinate human red blood cells (RBC) (Xu

and Chen, 1989)(Hanchun et al., 1991)(Ruvoën-Clouet et al., 1995), irrespective of their ABO phenotype (Ruvoën-Clouet et al., 2000), while not agglutinating erythrocytes from rabbits or other mammals (Oriol et al., 1992)(Ruvoën-Clouet et al., 2000). The interaction with human erythrocytes has been used for RHDV diagnosis and detection in tissues from victimized rabbits (Capucci et al., 1991)(Chasey, 1997), using the Haemagglutination (HA) test" (see Chapter I "The RHD laboratorial diagnosis", point "1.2.1.2 Antigen detection").

Rabbits were shown to express complex patterns of HBGAs on the duodenum surface (Breimer et al., 1979)(Oriol et al., 1992)(Miller-Podraza et al., 1997)(Nyström et al., 2011)(Leuthold et al., 2015). Several HBGA types are also weakly expressed in the trachea (Ruvoën-Clouet et al., 2000) and biliary ducts (Nyström et al., 2011)(Leuthold et al., 2015). At least four HBGAs were found to interact with RHDV strains, namely A, B and H type 2 and Lewis Y (Ruvoën-Clouet et al., 2000)(Nyström et al., 2011)(Leuthold et al., 2015).

1.3.2.3.1.1. HBGA binding pocket

A recent study by Leuthold et al. (2015) showed that the RHDV HBGA binding pocket is in a negatively charged patch on the side of the P domain and located at a dimeric interface. Residues from both monomers contribute to the HBGA binding which involved a network of direct hydrogen bonds and water-mediated interactions. An amino acid sequence alignment of different RHDV strains carried out in that study, indicated that the residues directly interacting with the ABH-fucose of the HBGAs (Asp_{472}, Asn_{474}, and Ser_{479}) were highly conserved among different RHDV P domains, suggesting that different RHDV strains also could bind HBGAs at the equivalent pocket (although amino acid variations surrounding the pocket may influence the interactions).

Structural analysis of the RHDV2 HBGA binding pocket performed by Leuthold et al. (2015) showed that the three conserved residues were located on a single P2 subdomain loop. Superposition of the unbound RHDV2 and RHDV2 P domain H2-tri complex revealed that the loop had slightly different conformations, especially between residues 477 and 482. The loop was found to be flexible, but how the flexible loop may have affected the HBGA binding interactions is yet to be clarified.

1.3.2.3.1.2. Resistance mechanisms to RHDV related to HBGA binding

This issue is addressed in Chapter III "RHD pathogenesis and prophylaxis", point "1.1.3. Resistance mechanisms to RHDV".

1.3.2.3.1.3. Shared human/rabbit ligands for RHDV

Classical RHDV infection has been shown to be rabbit specific, suggesting that other molecular elements not shared between rabbits and other mammals restrict its host range (Nyström et al., 2012). However, RHDV-RNA was detected in sympatric wild small mammals (Merchán et al., 2011) and in Iberian hares (Lopes et al., 2014), indicating that the species range of RHDV may not be as limited as previously believed. In addition, the recently emerged RHDV2 was detected in three different hare species (Camarda et al., 2014)(Puggioni et al., 2013)(Lavazza A, *personal communication*).

A relatively recent study indicates that attachment factors for RHDV are present on human cells, constituting potential points of entry for RHDV (Nyström et al., 2012). Attachment to HBGAs of human calicivirus strains represents the first step of the infection process (Nyström et al., 2012). This cross-species recognition of HBGAs in cells that may constitute points of RHDV entry into human cells, emphasises that more studies are needed to decode the molecular mechanisms involved in RHDV pathogenicity and to clarify its zoonotic potential (Nyström et al., 2012).

The barriers to viral emergence in novel host species are assumed to be difficult to overcome. Most cases of viral emergence represent transient "spill over" infections, in which only a few individuals of a novel host species acquire the new virus and without a sustained chain of transmission (Kitchen et al., 2011). A phylogenetic analysis showed that the colonization of new but related host species may represent the principle mode of macroevolution in RNA viruses and that, overall, caliciviruses exhibit a high level of host switching (Kitchen et al., 2011). Nevertheless, for primates and lagomorph reservoir hosts there was significant support for the clustering of viruses within the same reservoirs across the caliciviruses phylogeny (Kitchen et al., 2011).

Despite the evident evolutionary barriers to switching hosts, it is more likely that viruses will successfully escape niche overlap through allopatric rather than sympatric processes, although the precise mechanisms underlying this form of divergence are generally unknown (Kitchen et al., 2011). A host switch may require both less net movement through sequence space and cost less in terms of fitness than the substantial changes that may be necessary to acquire a new niche within the same host species. Kitchen et al. (2011) suggest that, although cross-species transmission and

emergence is normally regarded as an unusual mode of viral evolution, it may be a common form of RNA virus macroevolution.

1.3.4. RHD laboratorial diagnosis

The RHD laboratorial diagnosis is discussed in **Chapter I**. Sample preparation and direct and indirect detection methods are addressed. Direct methods include electron microscopy, antigen detection, molecular methods, immunohistochemical techniques "*in situ*" hybridization and viral isolation. Indirect methods refer to antibodies detection.

1.3.5. Information on sanitary status and importation recommendations regarding RHD

This information is given in **Chapter II.**

1.3.6. RHD Pathogenesis and prophylaxis

RHDV pathogenesis including disease forms and clinical manifestations, induced lesions, pathogenesis, mechanisms of resistance to RHDV and RHD prophylaxis is addressed in **Chapter III**.

1.3.7. RHD eco-epidemiology

RHD eco-epidemiology is reviewed in Chapter **IV.** The issues addressed in the chapter include susceptible hosts, viral survival in the environment, the impact of climatic factors, disease transmission, the effect of host related factors, the effect of population dynamics on the RHD impact and management strategies to enhance wild rabbits populations.

1.3.8. RHDV and RHDV2 genetic relations

This issue is addressed in **Chapter V**, where the RHDV and RHDV2 origin and evolution, genetic diversity and phylogeography are discussed. The non- or moderately pathogenic lagoviruses are also addressed in this chapter.

To better frame the reader in the comprehension of this chapter and of the studies included, a review on the methods used for phylogenetic analysis is provided at the beginning of the chapter.

1.3.9. Virus host co-evolution

The virus-host biological interaction is a continuous co-evolutionary process involving the host immune system and viral escape mechanisms (Lobo et al., 2009). During the co-evolution progression, the host and the virus evolve features to battle each other. While the first mounts defence mechanisms, the second develops counterstrategies to overcome those defences (Souiri et al., 2016). In these circumstances, hosts which develop strategies to avoid or limit virus infection are expected to be favoured by natural selection. Similarly, viruses which develop mechanisms to evade host natural defences are expected to be favoured as well (Lobo et al., 2009).

Although difficult to demonstrate rigorously in practice, host-pathogen co-evolution is considered a powerful determinant of the biology and genetics of infection and disease (Woolhouse et al., 2002) and implies very slow long-term evolutionary rates (Sharp and Simmonds, 2011). Evidence for host-virus co-evolution comes from a match between the phylogenetic trees of the viruses and their hosts (Lobo et al., 2009). Yet, if a virus has been co-diverging with its hosts, over the longer term, the virus phylogeny should be similar to that of the hosts but not necessarily identical (Sharp and Simmonds, 2011). This is because molecular clock approaches can underestimate, often by orders of magnitude, the ages of ancestral viruses and several viral families seem to be much older than previously realised (Sharp and Simmonds, 2011). Over this longer timescale, co-divergence of viruses with their hosts may have been much more frequent than suggested (Sharp and Simmonds, 2011).

In the long-term, co-evolution involves the origin of life forms, the evolution of host and vector, especially arthropods, kingdoms and families, and changes in biological diversity induced mainly by the last five great extinctions (Lovisolo et al., 2003). To study the European wild rabbit and RHDV co-evolution, the rabbit evolutionary history (already addressed in General Introduction, point 1.1) should be taken into account. In the medium term, the diversification of hosts and vectors is important, and in the short term, recent events, especially human-made, may have had a great impact on virus co-evolution (Lovisolo et al., 2003).

To understand and predict the virulence patterns of novel infections and reduce their impact, suitable models to study the mechanisms of disease emergence are critical (Holmes, 2013). The classical model of the evolution of virulence, even though the critical body of work occurred in the pre-genomic era, is the attenuation of myxoma virus (MYXV), family *Poxviridae*, genus *Leporipoxvirus*, following its introduction as a biological control agent for wild rabbit populations in Australia and Europe (Kerr et al., 2012). The intentional release of MYXV provided a unique

opportunity to study the initial spread and establishment of this pathogen as well as its co-evolution with the European wild rabbit, offering insights into how the virulence of a large DNA virus may evolve following a species jump (Kerr et al., 2012). The same kind of rare opportunity was provided later by the intentional release of RHDV in Australia, also as a biocontrol agent (Elsworth et al., 2014).

Given the major biological differences between both viruses, it was expected that RHDV and MYXV would follow different evolutionary trajectories (Elsworth et al., 2014). MYXV is a large double-stranded DNA genome, with several genes that may act as virulence determinants, mechanically transmitted by biting or blood sucking arthropod vectors such as fleas and mosquitoes and a reduction of virulence potentially maximises transmission, as the virus particles uptake occurs from lesions on live animals (Fenner and Ratcliffe, 1965) (Holmes, 2013). On the other hand, RHDV is a small, rapidly-replicating RNA virus, probably with a limited set of mutations controlling virulence (Kerr et al., 2012)(Holmes, 2013), that can be transmitted both orally by fomites or direct contact between rabbits (Morisse et al., 1991) or passively by scavenging insect vectors feeding on carcasses (Asgari et al., 1998)(McColl et al., 2002a)(Cooke, 2002) which facilitate the long distance transmission of the virus (Kovaliski et al., 2014)(Elsworth et al., 2014). Cadavers are the main source of virus in field transmission (Elsworth et al., 2014).

Regarding the canonical model of MYXV, initially the virus caused mortality rates of 99.8% (Fenner and Ratcliffe, 1965) but within a few years, and despite the ongoing release of virulent viruses, slightly attenuated MYXV strains came to dominate field populations (Kerr et al., 2012). Although these intermediate virulence strains still killed 90–99% of infected rabbits, their lower virulence allowed infected rabbits to survive longer, increasing the probability of transmission from skin lesions by mosquito and flea vectors (Fenner and Marshall, 1957)(Kerr et al., 2012). Simultaneously, natural selection acted on the wild rabbit population, resulting in the appearance of animals resistant to myxomatosis (Marshall and Fenner, 1958)(Kerr et al., 2012), probably in relation to an enhanced innate immune response allowing rabbits to mount an effective cellular immune response (Best and Kerr, 2000). With the attenuation of MYXV and developing genetic host resistance, rabbit populations in Australia began to recover (Elsworth et al., 2014). Parallel adaptive processes were observed on the European continent following the MYXV introduction (Fenner and Ratcliffe, 1965)(Kerr et al., 2012).

Studies on RHDV carried out in Australia also suggest that the virus became less effective in keeping wild rabbit numbers low and, in some populations, rabbit numbers returned to the pre-

RHDV levels (Elsworth et al., 2014). Initially, RHDV caused rabbit numbers to drop of up to 95% in many areas of Australia, but an evolutionary trend towards higher virulence was observed, contrarily to MXYV (Elsworth et al., 2014).

The RHDV evolutionary trend appears to be related to the fact that rabbit carcasses, rather than diseased animals, are the likely source of mechanical insect transmission, reflecting the selection of strains that multiply most efficiently and have the highest number of infectious viral particles in the liver (Elsworth et al., 2014). Despite this apparent evolutionary trend towards higher virulence, the causative viral mutations are unclear (Elsworth et al., 2014).

RHDV have evolved to cause higher mortality rates, shorter survival times, increased replication speed and higher virus loads in the livers of the succumbing rabbits (Elsworth et al., 2014). Also, the strains able to overcome the developing genetic resistance of Australian wild rabbits seem to have been favoured (Parkes et al., 2008), suggesting the virus evolution towards an increased virulence in response to higher host resistance.

The proportion of rabbits of all ages with antibodies to RHDV as well as the abundance of rabbits was found to increase across time since the virus introduction in Australia, evidencing a change in the rabbit–virus interaction (Parkes et al., 2008). The mechanisms for genetic resistance to RHDV are not fully clarified, but may be related with different HBGA phenotypes on the intestine of rabbits that differ in their ability to bind different RHDV strains that could contribute to a genetic resistance to RHDV at the population level (Nyström et al., 2011). It has been proposed that differential binding preferences of RHDV to HBGAs can convey partial resistance to certain strains of RHDV in both Australian and European wild rabbits (Nyström et al., 2011). Outbreaks survivors in wild populations showed an increased frequency of weak binding ABH phenotypes, indicating selection for host resistance depending on the circulating strain (Nyström et al., 2011). This evidence supports the role of HBGA in the virus epidemiology while suggesting that the virus is shaping the hosts' HBGA diversity (Nyström et al., 2011). This issue is addressed in Chapter III "RHD pathogenesis and prophylaxis", point "1.1.3.1 HBGA polymorphisms".

1.4. Tularaemia

This topic is presented next in the review article "**CL Carvalho**, L Zé-Zé, I Lopes de Carvalho, EL Duarte. 2014. Tularaemia: a challenging zoonosis. *Comp Immunol Infec Dis*. 37(2):85-96".

It is noteworthy that the published book chapter "Lopes de Carvalho I., **Carvalho C.L.**, Núncio M.S. 2012. Tularémia. In *Doenças Associadas a artrópodes vetores e roedores*. Núncio MS & Alves MJ (Eds). Ministério da Saúde. Instituto Nacional de Saúde Doutor Ricardo Jorge, IP. ISBN: 978-972-8643-90-4. pp 99-105", also reviews the theme in Portuguese and is provided in **Annex I**.

As the review article was published in 2014, information on specific issues that needed update is given after this article is presented providing the reader the state-of-the-art on the theme. More recent information's on the tularaemia laboratorial diagnosis and genotyping methods, epidemiology and on *Francisella*-like endosymbionts will be specifically addressed in the introduction of chapters VI, VII and VIII, respectively, preceding the studies.

Comparative Immunology, Microbiology and Infectious Diseases 37 (2014) 85–96

Contents lists available at ScienceDirect

Comparative Immunology, Microbiology and Infectious Diseases

journal homepage: www.elsevier.com/locate/cimid

Review

Tularaemia: A challenging zoonosis

C.L. Carvalho[a,b], I. Lopes de Carvalho[c], L. Zé-Zé[b], M.S. Núncio[b], E.L. Duarte[a,*]

[a] Institute of Mediterranean Agricultural and Environmental Science (ICAAM), School of Science and Technology ECT, University of Évora, Portugal
[b] Centre for Vectors and Infectious Diseases Research, National Health Institute Doutor Ricardo Jorge, Águas de Moura, Portugal
[c] Emergency Response and Bio-preparedness Unit, National Health Institute Doutor Ricardo Jorge, Lisbon, Portugal

ARTICLE INFO

Article history:
Received 16 October 2013
Received in revised form
28 December 2013
Accepted 4 January 2014

Keywords:
Tularaemia
Francisella tularensis
Zoonosis
Category A biowarfare agents
Wild animals

ABSTRACT

In recent years, several emerging zoonotic vector-borne infections with potential impact on human health have been identified in Europe, including tularaemia, caused by *Francisella tularensis*. This remarkable pathogen, one of the most virulent microorganisms currently known, has been detected in increasingly new settings and in a wide range of wild species, including lagomorphs, rodents, carnivores, fish and invertebrate arthropods. Also, a renewed concern has arisen with regard to *F. tularensis*: its potential use by bioterrorists. Based on the information published concerning the latest outbreaks, the aim of this paper is to review the main features of the agent, its biology, immunology and epidemiology. Moreover, special focus will be given to zoonotic aspects of the disease, as tularaemia outbreaks in human populations have been frequently associated with disease in animals.

© 2014 Elsevier Ltd. All rights reserved.

Contents

1. Introduction 86
2. Microbiology and phylogeography of *F. tularensis* 86
3. Epidemiology of *F. tularensis* 88
4. *F. tularensis* life cycle 89
5. Immunopathogenesis 89
6. Innate immune response 89
7. Acquired immune response 90
8. Clinical manifestations of tularaemia 90
   8.1. Humans 90
   8.2. Animals 91
9. Laboratory diagnosis 91
   9.1. Samples 91
   9.2. Culture 92
10. Microbiologic identification of *F. tularensis* 92
11. Serology 92
12. Molecular methods 92
13. Treatment 93
14. Vaccination 93

* Corresponding author at: ICAAM/ECT, Department of Veterinary Medicine, Apartado 94, 7002-554 Évora Codex, Portugal. Tel.: +351 266760859.
E-mail address: emld@uevora.pt (E.L. Duarte).

0147-9571/$ – see front matter © 2014 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.cimid.2014.01.002

15. Conclusions 94
Conflict of interest statement 94
Acknowledgements 94
References 94

1. Introduction

Seventy-five per cent of emerging infectious diseases are zoonotic [1]. Some wildlife species have been recognised as being major reservoirs for infectious diseases and the proximity of wildlife habitats and the existence of arthropod vectors with a wide geographical spread have rendered epidemiological cycles more complex [1].

Tularaemia is a zoonosis caused by the *Francisella tularensis* bacterium, which was first isolated in 1912 in Tulare County, California, by George McCoy and Charles Chapin [2–4]. Initially termed *Bacterium tularense*, it was allocated to a new genus and named *F. tularensis* in honour of the pioneer of research on the organism, Edward Francis [2,4]. Arthropod-borne transmission of tularaemia was first demonstrated by Francis in 1919 when he isolated the etiologic agent in a patient with "deer fly fever" [2,5,6].

Tularaemia was recognised as an important disease in the last century and since then there has been a growth in enthusiasm for research on this pathogen [7,8]. Interest has arisen with regard to *F. tularensis* as it has emerged in new locations, populations and settings, and increasingly figured in scientific research gauging its potential use in bioterrorism [7,9]. The European Centre for Disease Control and Prevention (ECDC) 2012 surveillance report refers a total of 891 confirmed cases of tularaemia in a number of European countries in 2010, with Sweden reporting the highest confirmed case rate, followed by Finland and Hungary [10]. Tularaemia is considered an unusual disease and the confirmed case rate in Europe has remained stable from 2006 to 2010. Recent outbreaks of tularaemia have occurred in several European countries, presented in Table 1, including the Czech Republic, Kosovo, Bulgaria, Germany, Sweden, Finland, Spain, Turkey, France and Norway [11–20]. Besides these outbreaks, sporadic case notifications have occurred in Austria, Estonia, Italy, Lithuania, Poland, Romania, Slovakia and the United Kingdom [10]. Although there are no reports of tularaemia for Denmark during this period, a confirmed case of the disease in a human was recorded there in 2003 [21]. In Portugal, the bacterium has been detected in the blood of an asymptomatic man and in a *Dermacentor reticulatus* tick by molecular methods [9].

2. Microbiology and phylogeography of *F. tularensis*

F. tularensis is one of the most virulent microorganisms currently known, while as few as ten microorganisms can cause potentially fatal disease in man and animals [7,22]. This high rate of infectivity has led the Centre for Disease Control and Prevention (CDC) to classify *F. tularensis* as a Category A biowarfare agent [23].

F. tularensis is a gram-negative, catalase-positive, pleomorphic and non-motile cocobacillus, characterised as a facultative intracellular pathogen that can grow within different types of cells including macrophages, hepatocytes and epithelial cells [2,22,24,25]. The cell wall of *F. tularensis* has an unusually high level of fatty acids with a unique profile for the genus, and wild strains have a lipid-rich capsule, with neither toxic nor immunogenic properties [2,5,6]. Capsule loss has been related to a decrease in virulence, although the viability or survival of the bacterium within neutrophils may remain unaltered.

F. tularensis is a *gamma* (γ)*-Proteobacteria* of the *Francisellaceae* family [2,4,22]. *F. tularensis* is the most common and pathogenic species and is formally divided into three subspecies with different pathogenicities and geographic distributions: *tularensis*, *holarctica* and *mediasiatica*. The species *Francisella novicida* is currently widely accepted as a fourth subspecies of *F. tularensis* [3,4,26–31], as it shares with *F. tularensis* an average of 99.2% nucleotide identity over a 1.1 Mbp of genome sequence [4,26,27,30]. However, some objections to the transfer of *F. novicida* to the subspecies rank of *F. tularensis* have been recorded, based on recent multiple genome sequencing results, which show divergent evolutions for *F. tularensis* and *F. novicida* populations. Therefore, separate species may be retained [32].

The *F. tularensis* subspecies *tularensis*, regarded as the most virulent subspecies and classified as Type A, occurs predominantly in North America [3,4,6,22,33]. Two distinct genetic sub-populations have been identified, AI and AII, which have different geographic distributions, hosts and vectors [3,4,6,26,30,34]. Sub-population AI has been additionally sub-divided into groups AIa and AIb [3,6,30,35]. The subspecies *holarctica*, related to milder forms of the disease and classified as Type B, occurs throughout the Northern Hemisphere [3,22,30,33]. Human infection with AIb strains usually have a fulminant clinical progression and are associated with high mortality rates, in contrast with infections by AIa and AII strains or Type B tularaemia [25,30,35]. Recently, this subspecies has also been detected in Tasmania, Australia [36]. Subspecies *mediasiatica* presents a similar virulence to subspecies *holarctica*, but its geographic distribution is restricted so far to Central Asia [26,33]. *F. novicida* is less virulent and has been isolated in North America, Australia and Thailand [3,26,29–33,37].

Based on a high degree of similarity between 16S rRNA gene sequences, other microorganisms have been classified as probable members of the *Francisellaceae* family; these include the *Francisella*-like endosymbionts or FLEs [6,8,38]. FLEs belong to a distinct phylogenetic clade from *F. tularensis* species [39]. The effect of FLEs, if any, on vector competency and in the transmission of *F. tularensis* by ticks is still unknown [6]. FLEs have a worldwide distribution and are vertically transmitted by hard and soft ticks of the genera *Amblyomma*, *Dermacentor*, *Ixodes* and *Ornithodoros* [39–42]. FLEs have been detected in ticks in North America

Table 1
Cases of tularaemia recorded in Europe.

European country	First report	Latest report	Suspected animal host	Tularaemia transmission to humans	Number of cases (year(s))	References
Czech Republic	1936 (humans and hares)	2000	Small mammals, particularly wild hares, rodents	Contact with tissues of infected animals, aerosols, contaminated food and water, tick bite	48 (2000)	[11,87–91]
Kosovo	1999–2000	2001/2002–2010	[a]	Ingestion of contaminated food or water	Ranging from 25 to 327 (2001–2010)	[12]
Bulgaria	1962 (muskrat)	1997, 2003–2005	Wild hares	Contaminated food and water, tick bite	285 (1997–2005) 24 (2003–2004)	[13,92]
Germany	1949	2004–2005	Wild hares, rodents	Contact with tissues of infected animals	39 (2004–2005)	[14,93,94]
Sweden	1931	2000–2005	[a]	Contaminated water, aerosols (farming), Mosquitoes	270 (2000) 698 (2003) 90 (2005)	[15,95–97]
Finland	[a]	2000, 2003, 2007	[a]	Mosquitoes, aerosols (farming), tick bite	50 (2007)	[16,21,76,97–99]
Spain	1997 (Human, wild hares)	2007	Small mammals (especially hares and rodents)	Aerosols, wild lagomorphs, canids, rodents, sheep, haematophagus vectors, crayfish	507 (2007)	[17,51,100,101]
Turkey	1936	2000, 2004–2010		Contaminated water	12 (2000) 61 (2004–2005) 12 (2005–2006) 40 (2010)	[18,102–107]
France	[a]	1997–2011	Wild hares	Contact with tissues of infected animals, aerosols, tick bite	144 (2007–2008) 51 (2011)	[19,108–110]
Norway	[a]	2003, 2005, 2007, 2011	Rodents (lemmings) and hares	Contaminated water or food	5 (2005) 9 (2007) 39 (2011)	[20,111,112]

[a] Information unavailable.

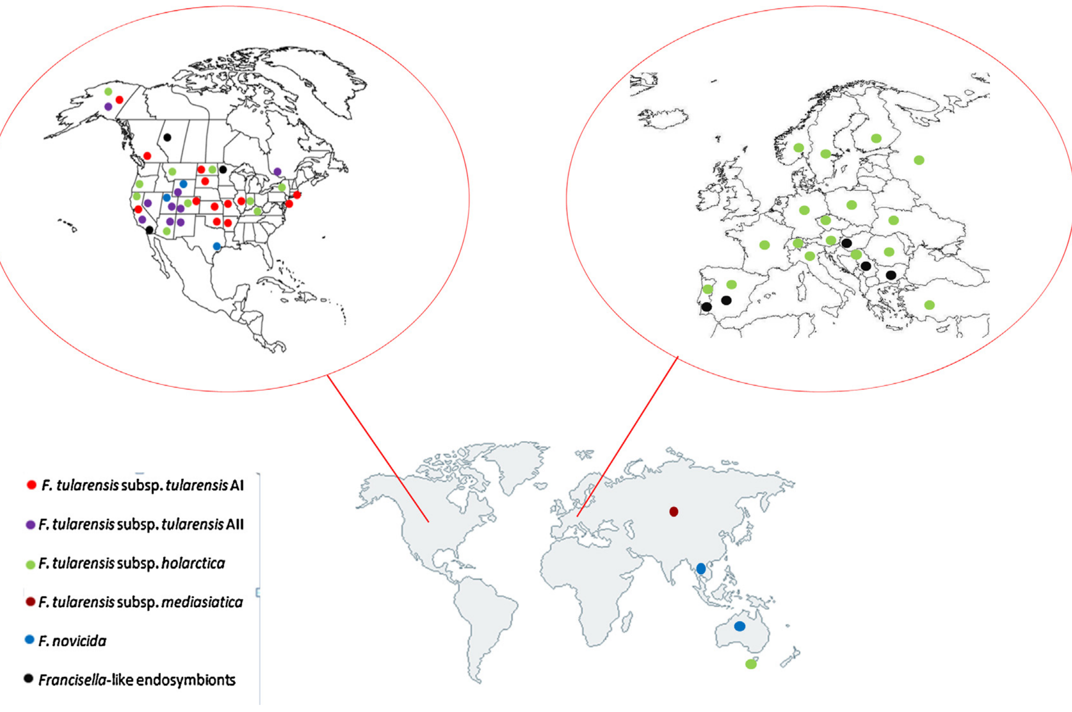


Fig. 1. Phylogeography of *F. tularensis* and *Francisella*-like endosymbionts.

(Texas, California, Minnesota), Canada (Alberta) and European countries such as Spain, Portugal, Hungary, Serbia and Bulgaria [38–45]. Their pathogenicity to humans is undetermined. They have recently been detected in free-living small mammals in Europe, suggesting the possible transmission of some FLE types from ticks to small mammals, although, to date, attempts to demonstrate it have failed [39,41,42,45,46]. The phylogeographic distribution of *F. tularensis* is given in Fig. 1; the geographic locations where FLEs have been detected in ticks are also indicated.

3. Epidemiology of *F. tularensis*

In nature, *F. tularensis* has been detected in a high number of wild species including lagomorphs, rodents, insectivores, carnivores, ungulates, marsupials, birds, amphibians, fish, and invertebrates [6,22,27,39,46–48].

Lagomorphs and rodents are considered as the main reservoirs of *F. tularensis* [6,22,46]. Wild lagomorphs, such as the European brown hare (*Lepus europaeus*), are thought to be suitable sentinels for *F. tularensis* and disease surveillance [46,47]. Recently, there have been serological evidences that foxes and raccoon dogs could also act as biological indicators for tularaemia [48].

Natural infections with *F. tularensis* have also been documented in different arthropods, although only a subset of these have been identified as important in *F. tularensis* transmission to humans. Still, few pathogens show the adaptability of *F. tularensis* to such a wide range of arthropod vectors capable of infection dissemination [6]. Arthropod found infected in nature include ticks of the genera *Amblyomma, Dermacentor*, *Ixodes* and *Ornithodoros*, mosquitoes of the genera *Aedes*, *Culex*, *Anopheles* and *Ochlerotatus excrucians*, and flies from the *Tabanidae* family (*Tabanus* spp., *Chrisozona* spp. and *Chrisops* spp.) [6,22,27,49]. Nevertheless, vector competence has only been demonstrated in ticks of the genera *Dermacentor* [35]. Tick-borne transmission of *F. tularensis* usually results in sporadic cases, although occasional outbreaks have also been reported [6]. Although regarded as merely mechanical vectors, mosquitoes have been associated with widespread epidemics of tularaemia and are capable of transient disease transmission [6,50]. Both ticks and mosquitoes may be infected in the larval phase. Transtadial transmission has been demonstrated in ticks although in mosquitoes evidences for transtadial transmission are only based in molecular methods [35,50]. Although transovarial transmission of *F. tularensis* in ticks was reported [2,6,51], a recent study in *Dermacentor variabilis* has proved otherwise [52]. Despite dissemination to ovaries and then to the oocytes, the pathogen was not recovered from the subsequently hatched larvae. Tabanid flies are regarded as mechanical vectors for *F. tularensis* and the long-term survival of this bacterium does not occur in these arthropods [6].

The epidemiologic characteristics of vector-borne tularaemia vary throughout the northern hemisphere and also within a given geographic location. This is thought to be related to the abundance of different vectors and host species. This could explain why, in the USA, Sweden, Finland and Russia, the arthropod bite is a common mode of transmission to humans, whilst in Western and Central Europe, contact with infected animals and the ingestion of contaminated food or water have been reported as more common transmission modes. Differences in transmission patterns have also been recorded within the USA: in western states, both ticks and deer flies are considered to be important vectors of tularémia, while in the east only ticks are considered relevant. In Sweden and Finland, mosquitoes have been identified as the primary vectors [6].

In Portugal, the role of ticks and small mammals in the transmission of tularaemia is still the subject of research. A collection of 4949 mosquitoes belonging to the genus *Culex* (63.97%), *Ochlerotatus* (35.34%), *Anopheles* (0.42%), *Culiseta* (0.14%) and a small number of *Aedes aegypti* females from the island of Madeira (0.12%) have been analysed, although all the results were found to be negative [53]. So far, this is in accordance with previous findings regarding the epidemiology characteristics of vector-borne tularaemia, suggesting that, in Portugal, mosquitoes have no role in the transmission of this disease. Ticks are thought to be the most important vectors of tularaemia in the majority of countries where tularaemia is endemic [53]. Nevertheless, major on-going research on tularaemia, aiming at gauging the overall impact of the disease in Portugal, is expected to throw further light on the main *F. tularensis* sources.

In endemic areas, tularaemia is a seasonal disease, with higher incidence in late spring, summer and autumn, occurring annually over a 5-year period or unreported for more than a decade. Often, the number of cases varies widely from 1 year to another, which is thought to be due to temperature or precipitation variability. However, the association between climactic conditions and tularaemia outbreaks has yet to be demonstrated [49]. *F. tularensis* has been found to be extremely resistant to environmental stress, surviving for weeks in soil, water and animal carcasses, at low temperatures [22].

Human tularaemia outbreaks are often preceded by animal outbreaks, particularly in wild lagomorphs and rodents. This is usually related to an increase in the numbers of these species, increasing the probability of exposure to infected animals [4,22,27,49].

The transmission of tularaemia to humans can occur either by direct contact with infected animals or indirectly due to arthropod vector bites, the ingestion of contaminated water, food or aerosols inhalation. Aerosols can be dispersed by ventilators, farming, and the deposition of contaminated hay, either intentionally or unintentionally [22]. Domestic dogs and cats can also transmit tularaemia to humans after contact with an infected animal, environment or infected ticks [54–56]. Person-to-person transmission has not been described so far [2,22,49,54].

Tularaemia has been reported to occur in any age group. Men tend to present a higher prevalence than women [2,49]. Professions that are prone to contact with reservoirs or arthropod vectors have been associated with a higher infection risk: these include laboratory technicians, hunters, farmers, veterinary surgeons, and anyone handling the flesh of infected animals [22,27].

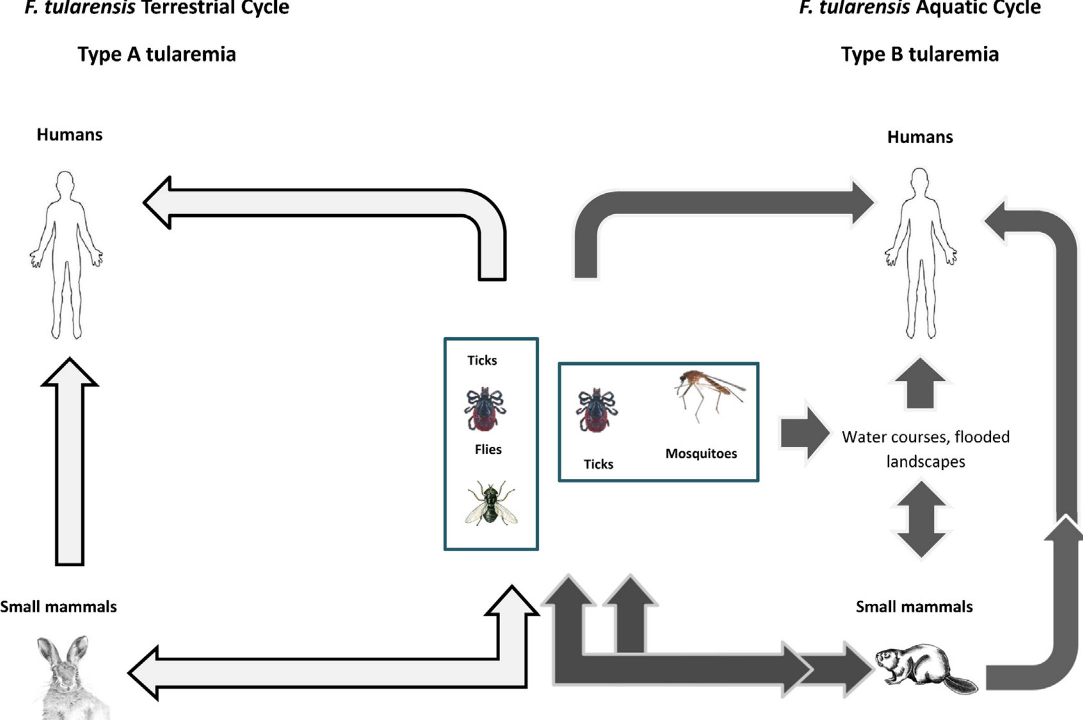


Fig. 2. Two life cycles of tularemia are recognised: the terrestrial cycle and the aquatic cycle. The terrestrial cycle is more commonly associated with Type A tularemias and the aquatic cycle with Type B.
Adapted from Akimana and Kway (2011) [113].

4. *F. tularensis* life cycle

Few pathogens show the adaptability of *F. tularensis* to varying vector, host and environmental conditions. Variations occur in local transmission cycles in association with differing ecologies. Both *F. tularensis* type A and type B are associated with different life cycles in which different animal hosts and arthropod vectors intervene [6]. Type A tularaemia is more commonly associated with the terrestrial cycle of the disease, with wild lagomorphs such as rabbits and hares acting as vertebrate hosts in which amplification of the agent occurs and where arthropods are disease-disseminating vectors [6,22,54,57]. Type B tularaemia is more frequently associated with the aquatic cycle, although outbreaks of tick-borne tularaemia involving subspecies *holarctica* have been reported [2,6,57]. In this life cycle, *F. tularensis* circulates in rodents such as beavers, muskrats and voles, and can be introduced in water courses from animal carcasses [6,22,27,54]. There is also evidence that *F. tularensis* can persist in water courses in association with amoebas [27,49,58]. Contaminated water can be the source of infection to humans, flies and mosquitoes [49]. An unusual waterborne outbreak of human tularaemia has been described in Spain associated with crayfish (*Procambarus clarkii*) caught in a contaminated freshwater stream. The crayfish acted as mechanical vectors, through mud- or water-contaminated carapaces, although the presence of *F. tularensis* in crayfish stomach and hepatopancreas could indicate their eventual role as hosts [51]. A diagrammatic representation of the terrestrial and aquatic cycles of tularaemia is shown in Fig. 2.

5. Immunopathogenesis

F. tularensis is a remarkable bacterial pathogen that can invade and multiply in a wide range of cell types [4,22,24,25,59]. Antigen-presenting cells (APC) such as macrophages or dendritic cells, appear to be the primary cell types targeted by the bacterium at the outset of infection [59]. The virulence of the bacterium is directly related to its capacity to replicate within the cytosol of infected cells [60]. *F. tularensis* clearly possesses several mechanisms by which it manipulates immunity. The bacterium evades detection at the point of entry in the host in three ways: (a) it has modified cell-surface structures that enable it to avoid interaction with host receptors that are associated with the induction of inflammation; (b) it targets cells that lack co-receptors which facilitate binding to receptors that might alert the host cell to invasion; (c) it utilises receptors that fail to initiate the production of pro-inflammatory cytokines [60].

6. Innate immune response

The entry of *F. tularensis* in macrophages occurs by means of a specific mechanism inherent to *Francisella* spp. [24]. The bacterium induces the macrophage to produce asymmetric spacious pseudopod loops in a “looping phagocytosis” process [4,61]. Uptake of *F. tularensis* is markedly

enhanced by serum opsonisation, which depends on serum intact complement factor C3 and host cell receptors (CR3), involving bacterial surface polysaccharides [4,62].

Utilisation of CR3 (and of mannose receptors of dendritic cells (MR) under non-opsonising conditions) is considered to be a fairly innocuous route for entry of *F. tularensis*, since it is not associated with the induction of signalling cascades that result in pro-inflammatory cytokines production. When opsonised by serum, *F. tularensis* binds iC3b and gains entry to host cells via the CR3 receptor [59].

The lipopolysaccharide (LPS) of subspecies *tularensis* is only moderately inflammatory and acts as an extremely weak toll-like receptor (TLR) 4 agonist stimulating a reduced production of pro-inflammatory cytokines [59,63]. These is attributed to the presence of only four acyl groups on the LPS that do not bind to the "LPS-binding proteins", subverting TLR4 recognition [4,25,59]. In addition to LPS, *F. tularensis* possesses two other TLR agonists [59]: Tul4 and FTT1103 lipoproteins. These interact with TLR2 and may alert the host cell for the presence of the bacterium prior to phagocytosis [4,25,59]. TLR2/myeloid differentiation primary response gene (88) (MyD88) signalling is essential for the production of pro-inflammatory cytokines and is critical for host defence against *Francisella* infection [24,61,63,64].

F. novicida has been used as a model organism to study immunity to *F. tularensis*. Nevertheless, *F. novicida* expresses a structurally distinct chemotype of LPS that is more pro-inflammatory in mice than the dominant LPS chemotype, and is expected to result in different inflammasome activations [25]. *F. novicida* escapes the phagossome and replicate in the cell cytosol where it is recognised by the inflammasome signalling system [24,25,60,64]. Inflammasome *stimuli* activate the protease cysteine aspartate-specific Caspase-1, promoting the release of potent pro-inflammatory cytokines responsible for cell apoptosis [24,60]. This results in *F. novicida* release from infected cells and enables the infection of new ones [24,60].

F. tularensis survival and replication within macrophages is enabled by a large set of virulence genes that include the "macrophage growth *locus*" (*mgl*) A and B and the "*Francisella Pathogenicity Island*", FPI [24]. FPI encodes for a putative type VI secretion system [4,8] and contains 19 genes that have been demonstrated as essential for intra-cellular growth and virulence [24]. Less virulent *F. novicida* presents only one copy of FPI in contrast with *F. tularensis* subspecies *tularensis* and *holarctica* that present two copies [4,24]. Genes within the FPI are regulated by *mgl*A [4]. Although current knowledge of the gene's functions is far from complete, this is one of the most active areas of *Francisella* research [8].

Following phagocytosis of opsonised *F. tularensis* by polymorphonuclear cells (PMN), the bacterium actively inhibits superoxide anion generation (ROS) via NADPH oxidase. This allows *F. tularensis* to evade the phagosome and persist in the cell cytosol. The contribution of polymorphonuclear cells seems to be related to the secretion of cytokines and chemokines that recruit effector cells to the infection site [25]. However, an excessive recruitment of neutrophils, modulated by an increase in metaloprotease-9 from the matrix, plays an important role in modulating leucocyte recruitment and seems to be directly related to *F. tularensis* pathogenesis [24,25].

Natural killer (NK) cells from the liver, spleen and lung also play an important role in the innate immune response, in particular by producing INF-α following primary infection by *F. tularensis* [25].

7. Acquired immune response

As *F. tularensis* is an intracellular pathogen, cellular immune response is believed to be the main defence mechanism. Memory effector T cells CD4+ and CD8+ are clearly important for the primary control of infection. These cells produce Type Th1 cytokines like INF-γ, TNF-α and IL-2 that are critical for the initial response to *F. tularensis* infection [25].

Although the role of humoral immunity in *F. tularensis* infection is believed to be less important, some studies have demonstrated the enhanced recovery of infected humans that have received hyper-immune serum [59]. Also, infection-specific IgM, IgA and IgG antibodies produced are good exposition indicators and may interfere with the ability of bacteria to infect host cells [25,49,59]. The contribution of B cells in defence is thought to be dependent on strain virulence [8,25]. Research on anti-*Francisella* antibodies targets is expected to allow for the identification of new diagnostic or reactive antigens and the development of vaccines [8].

Furthermore, *F. tularensis* is capable of influencing multiple pathways, and continued research into the specific mechanisms by which *F. tularensis* evades, modulates and suppresses the host immune response will improve our understanding of tularaemia pathogenesis and the regulation of host immunity [59].

8. Clinical manifestations of tularaemia

8.1. Humans

Relevant clinical disease has been reported with *F. tularensis* subsp. *tularensis* and *holarctica*. Clinical manifestations of tularaemia depend on strain virulence, infective dose and infection route, the extent of systemic involvement and host immune status [2,4,49]. The incubation period averages 3–5 days but ranges from 1 to 20 days. The disease has an acute onset, with the occurrence of fever (38–40 °C), chills, fatigue, generalised myalgia and headaches, resembling a flu-like syndrome [2,22,49]. The subspecies *tularensis* (Type A) causes severe disease, potentially fatal if untreated. The subspecies *holarctica* (Type B) causes less severe disease and fatalities are rare [49]. Depending on the route of infection, the following forms of the disease are described: ulceroglandular, glandular, oculoglandular, oropharyngeal, pneumonic, typhoidal and septic [22,49].

Ulceroglandular and glandular forms of the disease are the most common and frequently result from an arthropod bite or animal contact [2,4,49]. In ulceroglandular tularémia, a soft, painless ulcer develops at the inoculation

site and evolves to a scar [6,22]. This presentation is associated with fever, lymphadenopathy and, in Type A tularaemia, pneumonia and pleural effusion can occur [49]. In glandular tularaemia, the primary ulcer is unrecognisable [2,6,22,49].

Direct contamination of the eye through contaminated fingers, splashes or aerosols, may be followed by oculoglandular tularaemia. Unilateral conjunctivitis, with ulcers or papules in some patients, photophobia and epiphora are the main signs of this form of the disease [2,49].

Oropharyngeal tularaemia is acquired by means of contaminated food or water intake and aerosol inhalation [22]. It develops with ulcerative and exudative stomatitis and pharyngitis [49].

Pneumonic tularaemia occurs by means of contaminated aerosol inhalation but can also arise as a complication of any of the other disease forms by haematogenous generalisation [2,22,49]. Initial disease development is characterised by fever, cough, pleuritic chest pain and dyspnoea, along with other unspecific symptoms. Type A tularaemia is associated with significantly severer and more fulminant forms of pneumonia [2,49].

Typhoidal tularaemia refers to a systemic and febrile form of the disease in which no route of infection acquisition can be established [2,49].

Septic tularaemia is a severe and often fatal form of the disease that can occur as a complication of the ulceroglandular form in Type A tularaemia [22,49]. Patients can present unspecific and neurologic symptoms, and septic shock, SIRS (systemic inflammatory response syndrome), DIC (disseminated intravascular coagulation), haemorrhages, SARS (severe acute respiratory syndrome) and multiple organ failure [22,49]. In Type B tularaemia, complications of meningitis and septicaemia have only occasionally been described [49].

8.2. Animals

Clinical manifestations largely depend on the susceptibility of animal species to *F. tularensis* [49]. In wild animals, clinical signs of tularaemia are not well documented, and *post-mortem* findings are highly unspecific and include splenomegaly and punctual necrotic lesions in the liver and spleen [49,54].

In one experimental study in European brown hares (*Lepus europaeus*), clinical signs developed 1-day post-inoculation with a *F. tularensis* subspecies *holarctica* strain. These included fever, lethargy and anorexia. Two of the five hares in the study succumbed to the infection on days 5 and 9 following inoculation. Pathological findings included splenomegaly, diffuse spleen necrosis and focal liver necrosis with hepatocytes vacuolisation. The remaining three hares were euthanised and revealed no pathological lesions. Both bacterial culture and mouse inoculation test failed to produce *F. tularensis* isolation [46]. In a natural outbreak of tularaemia in brown hares in France, all eight hares involved presented splenomegaly, congestion and haemorrhagic lesions of several organs, tracheitis and bronchitis [65]. A similar study carried out in Hungary on European brown hares naturally infected with *F. tularensis* subspecies *holarctica* also showed very similar results [47].

In another study, 20 female New Zealand white rabbits (*Oryctolagus cuniculus*) were exposed to Type A tularaemia aerosols, with three different doses. Seven of them died while the others developed fever, anorexia and weight loss, with all infecting doses. Haematological findings in six rabbits included lymphopenia, monocytopenia and thrombocytopenia. A bibasilar pneumonia and gastrointestinal tract gas distension were the only radiological findings. Necropsy findings demonstrated hepatosplenomegaly with extensive spleen necrosis and small white nodules. Some of the rabbits presented nodular lesions in the lungs while others showed haemorrhagic lesions [66].

A situation of particular public health significance, given the risk of pet-to-human transmission, is associated with infected prairie dogs (*Cynomys ludovicianus*) sold as pets in the USA and exported internationally [67,68]. A ban was put in place in the European Union and other countries regarding the import of prairie dogs and other rodent species after the USA monkeypox outbreak in 2003 [68,69]. Wild-caught prairie dogs are particularly susceptible to environmental stress, such as capture, transit and crowding, which can enhance disease manifestations. Clinical signs include lethargy, dehydration and grossly enlarged cervical lymph nodes. Prairie dogs can produce specific antibodies against *F. tularensis* and survive tularaemia infection, suggesting their potential role as *F. tularensis* reservoirs in nature. Moreover, one study found that all seropositive animals harboured live infectious bacteria, suggesting persistent infection [67].

Tularaemia has also been described in domestic dogs and cats [49,55], which may be infected by means of arthropod bites, direct contact with infected animals, their ingestion, or contaminated aerosols [70,71].

Cats usually develop severe illness with unspecific clinical signs like fever, lethargy, prostration, vomiting and anorexia, dehydration, regional or generalised lymphadenopathy, splenomegaly, tongue and oropharyngeal ulceration and jaundice [49,72,73]. Pathological findings include multiple necrotic foci on the lymph nodes, spleen, liver and lungs. Frequently, panleukopenia with toxic degeneration of the neutrophils and hyperbilirubinaemia with bilirubinuria are present [73].

Dogs are less susceptible and rarely manifest signs of the disease [55,56]. Nevertheless, they can act as carrier hosts [70] and transmit the bacterium by means their fur after contact with contaminated dead animals or soil [74]. In most cases, infection is self-limiting and recovery is spontaneous. However, only few cases of natural infection in dogs have been reported [55,56].

9. Laboratory diagnosis

9.1. Samples

In humans, samples should preferably be collected before the onset of antibiotherapy and depend on the clinical form of the disease. Samples may include non-heparinised whole blood, serum, respiratory tract

secretions and washes, swabs from visible lesions, lymph node aspirates or biopsies, urine, and autopsy materials [49].

In animals, serum is the preferential sample for all disease forms, but plasma and dry blood on paper filters can also be used. Blood samples should be collected at least 14 days after the onset of the symptoms. Lymph nodes or bone marrow aspirates, organs (lung, liver, spleen) and cerebrospinal fluid can also be used [49].

In the context of an outbreak or epidemiologic studies, samples should include arthropod vectors as well as environmental samples like water, soil and rodent faeces [49,54].

9.2. Culture

Culture is the gold standard for *F. tularensis* and must be carried out in biosecurity level 3 facilities (BSL-3) [2,22,49]. *F. tularensis* is a fastidious microorganism. Optimal growth conditions occur at 37 °C and pH 6.9 [5,24]. Cysteine-enriched media, such as enriched chocolate agar (CA) or 9% cysteine heart agar with blood medium (CHAB) must be used for this purpose [22,49,54]. Growth in a CHAB medium enables the presumptive identification of *F. tularensis* by characteristic growth at 24–48 h of round and smooth green opalescent shiny colonies, 2–4 mm in diameter [4,22,27,49,54]. Antibiotic supplementation of CHAB is possible in order to optimise growth and inhibit contaminants [22,49,54]. For cultures made from blood, the use of the BACTEC™ (BD) system or equivalent, BacT/Alert™ (Biomérieux) is recommended [49,54]. Liquid media is not suitable for *F. tularensis* growth, even when supplemented with cysteine [4,27,54].

10. Microbiologic identification of *F. tularensis*

Basic biochemical tests provide a presumptive identification of isolates and may be further complemented by immunological and molecular methods. Some additional biochemical tests, such as the ability to ferment glucose or glycerol, or the presence of the citrulline ureidase pathway are useful for subtyping purposes [54].

The commercial Microlog Microstation™ System (Biolog Inc., Hayward, CA) based on the ability to ferment glucose has been successfully used for differentiating between subspecies *tularensis* and *holarctica* [54,67]. Also, the commercially available Microbial Identification System (MIS) and Library Generation System (LGS) (MIDI, Inc, Newark, NJ) enables cell-wall fatty-acid analysis and can be used for the identification of *Francisella* at the genus level. It has also enabled the identification of atypical *F. tularensis* strains lacking cysteine requirements [54,75].

Immune based techniques have also been employed for identification: immunoblot analysis and immunofluorescence microscopy, either from grown cultures or clinical samples [54].

11. Serology

Antibodies against *F. tularensis* reach detectable levels 10–20 days post-infection [49]. A fourfold increase in the titre between acute and convalescent sera or a titre of 1:160 or greater of agglutinating antibodies is considered for diagnostic purposes [2,27,54,76]. Titres peak at a level of 320–1280 and decline slowly [76]. Serologic methods include the whole-cell agglutination test (Widal's reaction), the tube agglutination test, microagglutination assays, haemagglutination, ELISA (*Enzyme-linked immunosorbent assay*) and immunoblot [2,22]. ELISA has repeatedly been more sensitive than agglutination assays, with the additional advantage of determining separately different antibody classes (IgM, IgG and IgA) [54].

A combination of a first ELISA screening test complemented by an immunoblot confirmatory test, with higher specificity, is the current recommended two-step approach for the serological diagnosis of tularaemia [54].

The same approach can be used for animals. Serology has a limited use in highly susceptible species since death usually precedes the development of specific antibodies [47]. However, in endemic areas, antibodies for *F. tularensis* are frequently detected in wild animals that have developed immunity, including foxes and coyotes. This seroconversion is suspected as being related to subspecies *holarctica* infection since infection by the subspecies *tularensis* is expected to be fatal [27,49].

12. Molecular methods

Molecular methods are valuable diagnostic tools whenever culture is either not possible or is negative [2,22,49]. Moreover, they reduce the high risk of laboratory-acquired infections over conventional biochemical typing [2,21,77].

During recent years, polymerase chain reaction (PCR)-based methods have been successfully used for the rapid identification and classification of *Francisella* isolates, with increased sensitivity and specificity [54,78]. However, false positive results related to non-pathogenic closely related *Francisella* subspecies, occurring naturally in the environment, may hamper species and subspecies identification [78].

Conventional PCR targets are *tul*4 and *fop*A genes, which encode for *F. tularensis* superficial membrane lipoproteins. Both protocols show a good level of sensitivity and reasonable specificity in *F. tularensis* detection and may be used in blood, tissue or aerosol samples [4,49,54]. PCR product specificity is confirmed by sequencing, reverse-line blotting (RLB) or restriction fragment-length polymorphism (RFLP) [54].

Real time PCR for *F. tularensis* detection has been developed, in particular, TaqMan™ (Applied Biosystems) real time PCR multiple assay shows high specificity and sensitivity using four target genes: *ISFtu*2, 23 *kDA*, *tul*4 and *fop*A [49,54]. Real-time PCR for the differentiation between the subspecies *tularensis* and *holarctica* is also now available [79].

Further discrimination has been achieved using high-resolution genotyping methods including pulse-field gel electrophoresis (PFGE), amplified fragment-length polymorphism (AFLP), ribotyping, 16S rDNA gene sequencing, canonic insertion deletions and paired-end sequence mapping [26,27,34,80]. Still, as *F. tularensis* exhibits highly

conserved genomic sequences among strains of diverse origin, genetic polymorphisms allowing for individual strain typing have been difficult to find [77]. As for other bacteria, more recent PCR-based techniques such as variable-number tandem repeats (VNTR), multiple-locus VNTR analysis (MLVA) and short-tandem repeats (STR) typing have been successfully used for identification at the subspecies level and for molecular epidemiology purposes [54,77,80]. One of the most discriminatory methods for the molecular subtyping of *F. tularensis* is MLVA, which consists of a series of VNTR *loci* that are PCR amplified via flanking primer sites and examined for size variation [79]. One MLVA system designed for *F. tularensis* is based on polymorphisms of 25 VNTR *loci*, Ft-M1 to Ft-M25. This MLVA typing system has a greater discriminatory power when applied to a worldwide set of *F. tularensis* isolates and provides accurate classification at the subspecies level [77]. This MLVA system has recently been improved by redesigning the subset of the 25 previously identified VNTRs to produce a new optimised, multiplexed MLVA system with a similar level of discrimination but with fewer time and cost requirements [79]. Ten of the previously described VNTR *loci* were selected based on their discrimination ability within the subspecies: Ft-M02, Ft-M03, Ft-M04, Ft-M05, Ft-M06, Ft-M010, FT-M20, FT-M22, Ft-M23 and Ft-M24. Locus Ft-M20 was split into two loci, Ft-M20A (which contains the originally described 12 bp repeat and is polymorphic across subspecies) and Ft-M20B (which contains the insertion with its 15 bp repeat and varies only among type A.II and *F. novicida* isolates) [79].

While providing discrimination among strains, VNTRs are unsuited for determining deeper phylogenetic relationships due to mutational saturation. In this case, more accurate and alternative markers should be used, such as whole-genome sequence single nucleotide polymorphism (SNPs) [79]. Additional studies have shown a remarkable degree of discrimination of the *F. tularensis* phylogenetic structure, using a combined analysis with canonical whole-genome SNPs for major clade typing, and MLVA for high-resolution typing [26,79]. In a different study, the combined analysis of insertion-deletion markers, for subspecies and major clade typing, along with MLVA, was used [80].

Microarrays have also allowed for the differentiation of the four *F. tularensis* subspecies and have been proven useful for pathogenicity and virulence marker identification [54].

13. Treatment

Tularaemia usually responds to antibiotic therapy. Historically, aminoglycosides have been the drugs of choice for humans. Although clinically effective, they are rarely used now due their ototoxicity and nephrotoxicity. Nevertheless, gentamicin has been used for treatment of pneumonic tularaemia and aminoglycosides are now generally used in the most serious cases. Chloramphenicol is effective but seldom the first choice due to its possible irreversible effects on haematopoiesis. Tetracyclines have been associated with high relapse rates on withdrawal. Fluoroquinolones, such as ciprofloxacin, have been shown to be highly effective in *per os* and are the best choice for uncomplicated tularaemia. Also, ciprofloxacin has proved suitable and effective in the treatment of tularaemia in children and pregnant women [4,49]. In domestic animals, gentamicin, enrofloxacin, doxycycline and chloramphenicol are referred to as therapeutic options for dogs [55,70]. In cats, there are reports of the use of doxycycline or enrofloxacin and amoxicilin-clavulanic acid as being beneficial in the early stages of the disease [81].

14. Vaccination

Currently, there is no available licensed vaccine against *F. tularensis* although an attenuated Type B strain, known as the Live Vaccine Strain (LVS) was developed in the United States during the 1950s and used to vaccinate military personnel and laboratory workers [4,49,82–84]. LVS failed to uniformly protect against pneumonic tularaemia and when delivered in high titres caused mild tularaemia as an undesirable side-effect [85].

One focus of current research work in the USA and in Europe is to develop a vaccine for protection against *F. tularensis* intentional release [49]. The restricted efficacy of the LVS has fostered extensive research with a view to providing alternative vaccine formulations, including the exploration of different live and killed attenuated strains and immunogenic components to produce subunit vaccines [4,82]. In view of its immunogenic antigens, an effort has been made to develop attenuated strains of SchuS4, a representative strain of Type A tularaemia, for vaccine production. In fact, between LVS and SchuS4 strains there are about 35 genes that encode for different protein sequences, whose functions are not well defined, and may represent important immunogenic antigens. Still, given the increased virulence of the SchuS4 strain, only a small number of bacteria should be required to generate effective protection against wild type *F. tularensis* [85]. A recently published study demonstrated that inoculation with low doses of specific attenuated mutants of the *F. tularensis* strain SchuS4 provided protection against parenteral and intranasal challenge with a fully virulent wild type SchuS4 strain [86]. This favours the role of T-cell memory response as a critical determinant of *F. tularensis* immunity, additionally to the humoral response. This feature is the basis of the challenges foreseen for vaccine development, aiming at identifying antigen determinants that elicit an effective cellular-mediated immune response [4,82,84,85]. Cell-mediated immunity was found to persist three decades after tularaemia vaccination. A recent study sought to identify the T-cell responses present in immune individuals in order to characterise *F. tularensis*-specific immune response [84,86]. The findings showed that the production of INF-γ, macrophage inflammatory protein (MIP)-1β and CD107a (lysosome-associated membrane protein 1 or LAMP-1) by peripheral blood mononuclear cells appeared to be a characteristic of protective immune responses and that a correlation exists between these parameters and immunity [84].

15. Conclusions

Several factors such as human demographics and behaviour, international travel and commerce, including the animal trade, climactic changes and microorganism adaptation, have a potential impact on disease ecology and the emergence of zoonosis. The same factors are thought to be related to the emergence of tularaemia. Special concerns regarding this bacterium exist in relation to its high infectivity, and easy dispersion through aerosols and contaminated water, which make it a potential bioterrorism weapon. Also, tularaemia presents a wide geographic distribution and has recently emerged in new settings, particularly in Europe. In Portugal, an on-going research project on tularaemia aims to increase our knowledge about the disease, particularly its impact in this country, which is still poorly understood, in view of the fact that there is little information available to risk population and health professionals, with the result that there is a possible underestimation of prevalence in man and animals. To this regard, efforts have been made by the National Institute of Health to increase awareness of the disease among risk populations, particularly hunters and health professionals. In accordance with the preliminary results, on-going research will further identify and characterise *F. tularensis* circulating strains and develop molecular and typing methods with increased sensitivity, specificity and discriminatory power. The role of autochthon wild lagomorphs in the *F. tularensis* life cycle, their involvement in animal-to-human transmission and their suitability as tularaemia sentinels will be accessed. Moreover, considering the economic and social relevance of hunting-related activities in this country, with very few studies having acknowledged its relation to zoonotic disease transmission risks, research into infection in game species is of major importance.

F. tularensis is also associated with a considerably wider range of hosts and vectors than most zoonotic pathogens, although there is little information on bacterium mechanisms for adaptation to such a wide diversity of arthropod vectors. Despite our increasing knowledge of tularaemia and its etiological agent, many aspects of *F. tularensis* biology and epidemiology need to be further examined, particularly its pathogenicity and virulence, vaccine development, and the specific mechanisms by which *F. tularensis* evades, modulates and suppresses the host immune response. As with any zoonotic emergent disease, the role of wild and domestic animals in *F. tularensis* epidemiology needs to be further evaluated, in particular, those which may act as reservoirs. Other epidemiologic data such as the population dynamics of susceptible animals, particularly lagomorphs and rodents in Europe, should be part of surveillance programmes, as they are thought to be directly associated with disease transmission patterns. From a public health perspective, disease surveillance in animals is crucial in order to prevent and monitor human outbreaks, particularly in endemic areas, where contact between humans and wildlife reservoirs or vectors is likely. Although tularaemia is not regarded as a common disease, and there is little awareness of the disease among health authorities and practitioners, its eventual future impact as an emergent zoonosis should not be neglected.

Conflict of interest statement

The authors declare that they have no conflict of interest.

Acknowledgements

We acknowledge the work carried out by Ana Sofia Santos, Fátima Amaro, Hugo Osório, Maria João Alves and Rita de Sousa from the Doctor Ricardo Jorge National Health Institute, I.P., Centre for Vectors and Infectious Diseases Research. Funding was provided by the Portuguese Science and Technology Foundation (FCT) (PTDC/SAU-ESA 104947/2008). C. Carvalho has been awarded a FCT PhD scholarship (SFRH/BD/79225/2011).

References

[1] Vorou RM, Papavassiliou VG, Tsiodras S. Emerging zoonosis and vector-borne infections affecting humans in Europe. Epidemiol Infect 2007;135(8):123–47.
[2] Mandell GL, Bennett JE, Dolin R. Mandell, Douglas, and Bennet's principles and practice of infectious diseases, vol. 2. Elsevier, Churchill Livingstone; 2005. p. 2674–83.
[3] Hansen CM, Vogler AJ, Keim P, Wagner DM, Hueffer K. Tularemia in Alaska, 1938–2010. Acta Vet Scand 2011;18(53):61.
[4] Oyston PC. *Francisella tularensis*: unraveling the secrets of an intracellular pathogen. J Med Microbiol 2008;57(Pt. 8):921–30.
[5] Collier L, Barlows A, Sussman M. Topley and Wilson's Microbiology and Microbial Infections, vol. 2; 1998. p. 1347–9.
[6] Petersen JM, Mead PS, Schriefer ME. *Francisella tularensis*: an arthropod-borne pathogen. Vet Res 2009;40(2):7.
[7] Petersen JM, Schriefer ME. Tularemia: emergence/re-emergence. Vet Res 2005;36(3):455–67.
[8] Sjöstedt A. Special Topic on *Francisella tularensis* and tularemia. Front Microbiol 2011;2:86.
[9] de Carvalho I, Escudero R, Garcia-Amil C, Falcão H, Anda P, Núncio MS. *Francisella tularensis*, Portugal. Emerg Infect Dis 2007;13(4):666–7.
[10] European Center for Disease Control and Prevention. Annual Epidemiologic Report. Reporting on 2010 surveillance data and 2011 epidemic intelligence data. Stockholm: ECDC; 2013. p. 119–21.
[11] Pazdiora P, Morávková I, Nocarová D, Velkoborská M, Valecková K. A water-borne epidemic of tularemia in Chlumcany. Epidemiol Mikrobiol Imunol 2002;51(1):23–5.
[12] Grunow R, Kalaveshi A, Künh A, Mulligi-Osmani G, Ramadani N. Surveillance of tularemia in Kosovo, 2001 to 2010. Euro Surveill 2012;17(28), pii:20217.
[13] Kantardjiev T, Ivanov I, Velinov T, Padeshki P, Popov B, Nenova R, et al. Tularemia outbreak, Bulgaria, 1997–2005. Emerg Infect Dis 2006;12(4):678–80.
[14] Hauri AM, Hofstetter I, Seibold E, Kaysser P, Eckert J, Neubauer H, et al. Investigating an airborne tularemia outbreak, Germany. Emerg Infect Dis 2010;16(2):238–43.
[15] Wik O. Large tularaemia outbreak in Värmland, central Sweden, 2006. Euro Surveill 2006;11(9):E060921.1.
[16] Jounio U, Renko M, Uhari M. An outbreak of holarctica-type tularemia in pediatric patients. Pediatr Infect Dis J 2010;29(2):160–2.
[17] Allue M, Sopeña CR, Gallardo MT, Mateos L, Vian E, Garcia MJ, et al. Tularemia outbreak in Castilla y León, Spain, 2007: an update. Euro Surveill 2008;13(32), pii:18948.
[18] Dikici N, Ural O, Sümer S, Oztürk K, Albayrak Yigit O, Katlanir E, et al. Tularemia in Konya region, Turkey. Mikrobiyol Bul 2012;46(2):225–35.
[19] Decors A, Lesage C, Jourdain E, Giraud P, Houbron P, Vanhem P, et al. Outbreak of tularemia in brown hares (*Lepus europaeus*) in France, January to March 2011. Euro Surveill 2011;16(28), pii:19913.

[20] Larssen KW, Afset JE, Heier BT, Krogh T, Handeland K, Vikoren T, et al. Outbreak of tularemia in central Norway, January to March 2011. Euro Surveill 2011;16(13):19828.
[21] Byström M, Böcher S, Magnusson A, Prag J, Johansson A. Tularemia in Denmark: identification of a *Francisella tularensis* subsp., holarctica strain by real time PCR and high-resolution typing by multiple-locus variable number tandem repeat analysis. J Clin Microbiol 2005;43(10):5355–8.
[22] Lopes de Carvalho I, Núncio MS, David de Morais J. Tularémia. Acta Med Port 2009;3:281–90.
[23] CDC, www.Emergency.cdc.gov/agent/agentlist-category.asp
[24] Foley JE, Nieto NC. Tularemia. Vet Microbiol 2010;140(3–4):332–8.
[25] Cowley SC, Elkins KL. Immunity to *Francisella*. Front Microbiol 2011;2:26.
[26] Vogler AJ, Birdsell D, Price LB, Bowers JR, Beckstrom-Sternberg SM, Auerbach RK, et al. Phylogeography of *Francisella tularensis*: global expansion of a highly fit clone. J Bacteriol 2009;191(8):2474–84.
[27] Ellis J, Oyston PCF, Green M, Titball RW, Tularemia. Clin Microbiol Rev 2002;15:629–46.
[28] Huber B, Escudero R, Busse HJ, Seibold E, Scholz HC, Anda P, et al. Description of *Francisella hispaniensis* sp.*nov*., isolated from human blood, reclassification of *Francisella novicida* (Larson et al., 1955) Olsufiev et al., 1959 as *Francisella tularensis* subspecies *novicida* comb. *nov*. and emended description of the genus *Francisella*. Int J Syst Evol Microbiol 2010;60(Pt. 8):1887–96.
[29] Brett M, Doppalapudi A, Respicio-Kingry LB, Myers D, Husband B, Pollard K, et al. *Francisella novicida* bacteremia after a near-drowning accident. J Clin Microbiol 2012;50(8):2826–9.
[30] Birdsell DN, Stewart T, Vogler AJ, Lawaczech E, Diggs A, Sylvester TL, et al. *Francisella tularensis* subsp. *novicida* isolated from a human in Arizona. BMC Res Notes 2009;2:223.
[31] Siddaramappa S, Challacombe JF, Petersen JM, Pillai S, Hogg G, Kuske CR. Commom ancestry and novel genetic traits of *Francisella novicida*-like isolates from North America and Australia as revealed by comparative genomic analysis. Appl Environ Microbiol 2011;77(15):5110–22.
[32] Johansson A, Celli J, Conland W, Elkins KL, Forsman M, Keim PS, et al. Objections to the transfer of *Francisella novicida* to the subspecies rank of *Francisella tularensis*. Int J Syst Evol Microbiol 2010;60(Pt. 8):1717–8.
[33] Whipp MJ, Davis JM, Lum G, de Boer J, Zhou Y, Bearden SW, et al. Characterization of a *novicida*-like subspecies of *Francisella tularensis* isolated in Australia. J Med Microbiol 2003;52(Pt. 9):893–942.
[34] Fey PD, Dempsey MM, Olson ME, Chrustowski MS, Engle JL, Jay JJ, et al. Molecular analysis of *Francisella tularensis* subspecies *tularensis* and *holarctica*. Am J Clin Pathol 2007;128(6):926–35.
[35] Reese SM, Dietrich G, Dolan MC, Sheldon SW, Piesman J, Petersen JM, et al. Transmission dynamics of *Francisella tularensis* subspecies and clades by nymphal *Dermacentor variabilis* (Acari: Ixodidae). Am J Trop Med Hyg 2010;83(3):645–52.
[36] Jackson J, McGregor A, Cooley L, Ng J, Brown M, Ong CW, et al. *Francisella tularensis* subspecies *holarctica*, Tasmania, Australia, 2011. Emerg Infect Dis 2012;18(9):1484–6.
[37] Leelaporn A, Yongyod S, Limsrivanichakorn S, Yungyuen T, Kiratisin P. *Francisella novicida* bacteremia, Thailand. Emerg Infect Dis 2008;14(12):1935–7.
[38] Kugeler KJ, Gurfield N, Creek JG, Mahoney KS, Versage JL, Petersen JM. Discrimination between *Francisella tularensis* and *Francisella*-like endosymbionts when screening ticks by PCR. Appl Environ Microbiol 2005;71(11):7594–7.
[39] Escudero R, Toledo A, Gil H, Kovácsová K, Rodigues-Vargas M, Jado I, et al. Molecular method for discrimination between *Francisella tularensis* and *Francisella*-like endosymbionts. J Clin Microbiol 2008;46(9):3139–43.
[40] De Carvalho IL, Santos N, Soares T, Zé-Zé L, Núncio MS. *Francisella*-like endosymbiont in *Dermacentor reticulatus* collected in Portugal. Vector Borne Zoonotic Dis 2011;11(2):185–8.
[41] Ivanov IN, Mitkova N, Reye AL, Hübschen JM, Vatcheva-Dobrevska RS, Dobreva EG, et al. Detection of new *Francisella*-like endosymbionts in *Hyalomma* spp and *Rhipicephalus* spp. (Acari: Ixodidae) from Bulgaria. Appl Environ Microbiol 2011;77(15):5562–5.
[42] Dergousoff SJ, Chilton NB. Association of different genetic types of *Francisella*-like organisms with the rocky mountain wood tick (*Dermacentor andersoni*) and the American dog tick (*Dermacentor variabilis*) in localities near their northern distributional limits. Appl Environ Microbiol 2012;78(4):965–71.
[43] Scoles GA. Phylogenetic analysis of the *Francisella*-like endosymbionts of Dermacentor ticks. J Med Entomol 2004;41(3): 277–86.
[44] Sréter-Lancz Z, Széll Z, Sréter T, Márialigeti K. Detection of a novel *Francisella* in *Dermacentor reticulatus*: a need for careful evaluation of PCR-based identification of *Francisella tularensis* in Eurasian ticks. Vector Borne Zoonotic Dis 2009;9(1):123–6.
[45] Baldridge GD, Scoles GA, Burkhardt NY, Schloeder B, Kurtti TJ, Munderloh UG. Transovarial transmission of *Francisella*-like endosymbionts and *Anaplasma phagocytophilum* variants in *Dermacentor albipictus* (Acari: Ixodidae). J Med Entomol 2009;46(3):625–32.
[46] Gyuranecz M, Szeredj L, Makrai L, Fodor L, Mészáros AR, Szépe B, et al. Tularemia of Brown European Brown Hare (*Lepus europaeus*): a pathological, histopatological and immunohistochemical study. Vet Pathol 2010;47(5):958–63.
[47] Bandouchova H, Pohanka M, Vlckova K, Damkova V, Peckova L, Sedlackova J, et al. Biochemical responses and oxidative stress in *Francisella tularensis* infection: a European brown hare model. Acta Vet Scand 2011;53:2.
[48] Kuehn A, Shulze C, Kutzer P, Probst C, Hlinak A, Ochs A, et al. Tularaemia seroprevalence of captured and wild animals in Germany: the fox (*Vulpes vulpes*) as a biological indicator. Epidemiol Infect 2013;141(4):833–40.
[49] World Health Organization working group on Tularaemia. WHO Guidelines on Tularaemia; 2007, 115p.
[50] Lundström JO, Andersson AC, Bäckman S, Schäfer ML, Forsman M, Thelaus J. Transstadial transmission of *Francisella tularensis* holarctica in mosquitos, Sweden. Emerg Infect Dis 2011;17(5):794–9.
[51] Anda P, Segura del Pozo J, Díaz García JM, Escudero R, García Peña FJ, López Velasco MC, et al. Waterborne outbreak of tularemia associated with crayfish fishing. Emerg Infect Dis 2011;7(3 Suppl.):575–82.
[52] Mani RJ, Reichard MV, Morton RJ, Kocan KM, Clickenbeard KD. Biology of *Francieslla tularensis* subspecies *holarctica* live vaccine strain in the tick vector *Dermacentor variabilis*. PLoS ONE 2012;7(4):e35441.
[53] Carvalho CL, Zé-Zé L, Duarte EL, Núncio MS, de Carvalho IL. Screening of mosquitoes as vectors of *F. tularensis* in Portugal. In: 7th International Conference on tularemia. 2012.
[54] Splettstoesser WD, Tomaso H, Al Dahouk S, Neubaeur H, Schuff-Werner P. Diagnostic procedures in tularemia with special focus on molecular and immunological techniques. J Vet Med B Infect Dis Vet Public Health 2005;52(6):249–61.
[55] Meinkoth KR, Morton RJ, Meinkoth JH. Naturally occurring tularemia in a dog. J Am Vet Med Assoc 2004;225(4):545–7.
[56] Gustafson BW, DeBows LJ. Tularemia in a dog. J Am Anim Hosp Assoc 1996;32(4):339–41.
[57] Zarrella TM, Singh A, Bitsaktsis C, Rahman T, Sahay B, Feustel PJ, et al. Host-adaptation of *Francisella tularensis* alters the bacteruim' surface-carbohydrates to hinder effectors of innate and adaptive immunity. PLoS ONE 2011;6(7):e22335.
[58] Abd H, Johansson T, Golovliov I, Sandström G, Forsman M. Survival and growth of *Francisella tularensis* in *Acanthamoeba castellanii*. Appl Environ Microbiol 2003;69(1):600–6.
[59] Bosio CM. The subversion of the immune system by *Francisella tularensis*. Front Microbiol 2011;2:9.
[60] Henry T, Brotcke A, Weiss DS, Thompson LJ, Monack DM. Type I interferon signaling is required for activation of the inflammasome during *Francisella* infection. J Exp Med 2007;204(5):987–94.
[61] Cremer TJ, Butchar JP, Tridandapani S. *Francisella* subverts innate immune signaling: focus on PI3k/Akt. Front Microbiol 2011;5:13.
[62] Chong A, Celli J. The *Francisella* intracellular life cycle: toward molecular mechanisms of intracellular survival and proliferation. Front Microbiol 2010;1:138.
[63] Jones JW, Broz P, Monack DM. Innate immune recognition of *Francisella tularensis*: activation of type-I interferons and the inflammasome. Front Microbiol 2011;2:16.
[64] Jones CL, Weiss DS. TLR2 signaling contributes to rapid inflammasome activation during *F. novicida* infection. PLoS ONE 2011;6(6):e20609.
[65] Decors, Lesage C, Jourdain E, Giraud P, Houbron P, Vanhem P, et al. Outbreak of tularemia in brown hares (*Lepus europaeus*) in France, January to March 2011. Euro Surveill 2011;16(28), pii:19913.
[66] Reed DS, Smith L, Dunsmore T, Trichel A, Ortiz LA, Cole KS, et al. Pneumonic tularemia in rabbits resembles the human disease as illustrated by radiographic and hematological changes after infection. PLoS ONE 2011;6(9):e24654.
[67] Petersen JM, Schriefer ME, Carter LG, Zhou Y, Sealy T, Bawiec D, et al. Laboratory analysis of tularemia in wild-trapped, commercially traded prairie dogs, Texas, 2002. Emerg Infect Dis 2004;10(3):419–25.

[68] Avashia SB, Petersen JM, Lindley CM, Schriefer ME, Gage KL, Cetron M, et al. First reported prairie dog-to-human transmission, Texas, 2002. Emerg Infect Dis 2004;10(3):483–6.
[69] European Commission bans import of prairie dogs from the US and rodents and squirrels from sub-Saharan Africa. Euro Surveill 2003;7(25), pii:2243.
[70] Feldman KA. Tularemia. J Am Vet Med Assoc 2003;31(6):725–30.
[71] Woods JP, Panciera RJ, Morton RJ, Lehenbauer TW. *Feline tularemia*, vol. 20. The Compendium on continuing education for the practicing veterinarian; 1998. p. 442–57.
[72] Woods JP, Crystal MA, Morton RJ, Panciera RJ. Tularemia in two cats. J Am Vet Med Assoc 1998;212(1):81–3.
[73] Baldwin CJ, Panciera RJ, Morton RJ, Cowell AK, Waurzyniak BJ. Acute tularemia in three domestic cats. J Am Vet Med Assoc 1991;199(11):1602–5.
[74] Siret V, Barataud D, Prat M, Vaillant V, Ansart S, Coustumier AL, et al. An outbreak of airborne tularemia in France, August 2004. Euro Surveill 2006;11:58–60.
[75] Bernard K, Tessier S, Winstanley J, Chang D, Borczyk A. Early recognition of atypical *Francisella tularensis* strains lacking a cysteine requirement. J Clin Microbiol 1994;32(2):551–3.
[76] Tärnvik A, Berglund L. Tularemia. Eur Respir J 2003;21(2):361–73.
[77] Johansson A, Farlow J, Larsson P, Dukerich M, Chambers E, Byström M, et al. Worldwide genetic relationships among *Francisella tularensis* isolates determined by multiple-locus variable-number tandem repeat analysis. J Bacteriol 2004;186(17):5808–18.
[78] Ahlinder J, Ohrman C, Svensson K, Lindgren P, Johansson A, Forsman M, et al. Increased knowledge of *Francisella* genus diversity highlights the benefits of optimized DNA-based assays. BMC Microbiol 2012;12(1):220.
[79] Vogler AJ, Birdsell D, Wagner DM, Keim P. An optimized, multiplexed multi-locus variable-number tandem repeat analysis system for genotyping *Francisella tularensis*. Lett appl Microbiol 2009;48(1):140–4.
[80] Larsson P, Svensson K, Karlsson L, Guala D, Granberg M, Forsman M, et al. Canonical insertion–deletion markers for rapid DNA typing of *Francisella tularensis*. Emerg Infect Dis 2007;13(11):1725–32.
[81] Valentine BA, DeBey BM, Sonn RJ, Stauffer LR, Pielstick LG. Localized cutaneous infection with *Francisella tularensis* resembling ulceroglandular tularemia in a cat. J Vet Diagn Invest 2004;16(1):83–5.
[82] Zvi A, Rotem S, Cohen O, Shafferman A. Clusters versus affinity-based approaches in *F. tularensis* whole genome search of CTL epitopes. PLoS ONE 2012;7(5):e36440.
[83] Kilmury SL, Twine SM. The *Francisella tularensis* proteome and its recognition by antibodies. Front Microbiol 2011;1:143.
[84] Eneslätt K, Normark M, Björk R, Rietz C, Zingmark C, Wolfraim LA, et al. Signatures of T cells as correlates of immunity to *Francisella tularensis*. PLoS ONE 2012;7(3):e32367.
[85] Rockx-Brouwer D, Chong A, Wehrly TD, Child R, Crane DD, Celli J, et al. Low dose vaccination with attenuated *Francisella tularensis* strain SchuS4 mutants protects against tularemia independent of the route of vaccination. PLoS ONE 2012;7(5):e37752.
[86] Eneslätt K, Rietz C, Rydén P, Stöven S, House RV, Wolfraim LA, et al. persistence of cell mediated immunity three decades after vaccination with the live vaccine strain of *Francisella tularensis*. Eur J Immunol 2011;41(4):974–80.
[87] Tomake E. Tularemia in Czechoslovakia and Austria during 1936 and 1937. Am J Public Health Nations Health 1937;27(5):443.
[88] Pikula J, Treml F, Beklová M, Holesovska Z, Pikulová J. Ecological conditions of natural foci of tularemia in the Czech Republic. Eur J Epidemiol 2003;18(11):1091–5.
[89] Cerný Z. Changes of the epidemiology and the clinical picture of tularemia in Southern Moravia (The Czech Republic) during the period 1936–1999. Eur J Epidemiol 2001;17(7):637–42.
[90] Cerný Z. Tularemia – history, epidemiology, clinical aspects, diagnosis and therapy. Cas Lek Cesk 2002;141(9):270–5.
[91] Treml F, Hubálek Z, Halouzka J, Juricová Z, Hunady M, Janík V. Analysis of the incidence of tularemia in the Breclav District 1994–1999. Epidemiol Mikrobiol Imunol 2001;50(1):4–9.
[92] Komitova R, Nenova R, Padeshki P, Ivanov I, Popov V, Petrov P. Tularemia in Bulgaria 2003–2004. J Infect Dev Ctries 2010;4(11):689–94.
[93] Kaysser P, Seibold E, Mätz-Rensing K, Pfeffer M, Essbauer S, Splettstoesser WD. Re-emergence of tularemia in Germany: presence of *Francisella tularensis* in different rodent species in endemic areas. BMC Infect Dis 2008;8:157.
[94] Müller W, Bocklisch H, Schüler G, Hotzel H, Neubauer H, Otto P. Detection of *Francisella tularensis* subsp. *holarctica* in a European brown hare (*Lepus europaeus*) in Thuringia, Germany. Vet Microbiol 2007;123(1–3):225–9.
[95] Svensson K, Bäck E, Eliasson H, Berglund L, Granberg M, Karlsson L, et al. Landscape epidemiology of tularemia outbreaks in Sweden. Emerg Infect Dis 2009;15(12):1937–47.
[96] Payne L, Arneborn M, Tegnell A, Giesecke J. Endemic tularemia, Sweden 2003. Emerg Infect Dis 2005;11(9):1440–2.
[97] Eliasson H, Lindbäck J, Nuorti JP, Arneborn M, Giesecke J, Tegnell A. The 2000 tularemia outbreak: a case–control study of risk factors in disease-endemic and emergent areas, Sweden. Emerg Infect Dis 2002;8(9):956–60.
[98] Seppänen M. Insect-borne diseases and insect bites in Finland. Duodecim 2011;127(13):1393–400.
[99] Rydén P, Björk R, Schäfer ML, Lundström JO, Petersén B, Lindblom A, et al. Outbreaks of tularemia in a boreal forest region depends on mosquito prevalence. J Infect Dis 2012;205(2):297–304.
[100] Martín C, Gallardo MT, Mateos L, Vián E, García MJ, Ramos J, et al. Outbreaks of tularemia in Castilla y León, Spain. Euro Surveill 2007;12(11):E071108.1.
[101] M.Pérez-Castreillón JL, Bachiller-Luque P, Martín-Luquero M, Mena-Martín FJ, Herreros V. Tularemia epidemic in northwestern Spain: clinical description and therapeutic response. Clin Infect Dis 2001;33(4):573–6.
[102] Gurcan S, Karabay O, Karadenizli A, Karagol C, Kantardjiev T, Ivanov IN. Characteristics of the Turkish isolates of *Francisella tularensis*. Jpn J Infect Dis 2008;61(3):223–5.
[103] Bayhan-Tas GI, Tanir G, Celebi B. Two cases of glandular tularemia from Turkey. Turk J Pediatr 2012;54(2):203–6.
[104] Yesilyurt M, Kilic S, Celebi B, Gül S. Tularemia: are hunters really a risk group? Mikrobiol Bul 2012;46(1):153–5.
[105] Celebi G, Baruönü F, Ayoglu F, Cinar F, Karadenizli A, Ugur MB, et al. Tularemia a re-emerging disease in northwest Turkey: epidemiological investigation and evaluation of treatment responses. Jpn J Infect Dis 2006;59(4):229–34.
[106] Ozdemir D, Sencan I, Annakkaya AN, Karadenizli A, Guclu E, Sert E, et al. Comparison of the 2000 and 2005 outbreaks of tularemia in the Duzce region of Turkey. Jpn J Infect Dis 2007;60(1):51–2.
[107] Sahin M, Atabay HI, Bicakci Z, Unver A, Otlu S. Outbreaks of tularemia in Turkey. Kobe J Med Sci 2007;53(1–2):37–42.
[108] Maurin M, Pelloux I, Brion JP, Del Banõ JN, Picard A. Human tularemia in France, 2006–2012. Clin Infect Dis 2011;53(10):e133–41.
[109] Fournier PE, Bernabeu L, Schubert B, Mutillod M, Roux V, Raoult D. Isolation of *Francisella tularensis* by centrifugation of shell vial cell culture from an inoculation eschar. J Clin Microbiol 1998;36(9):2782–3.
[110] Li W, Raoult D, Rolain JM, La Scola B. Evidence of circulation of an epidemic strain of *Francisella tularensis* in France by multispacer typing. Eur J Clin Microbiol Infect Dis 2011;30(9):1135–8.
[111] Brantsaeter AB, Krogh T, Radtke A, Nygard K. Tularaemia outbreak in northern Norway. Euro Surveill 2007;12(3):E070329.2.
[112] Hagen IJ, Aandahl E, Hasseltvedt V. Five case histories of tularaemia infection in Oppland and Hedmark Counties, Norway. Euro Surveill 2005;10(3):E050317.4.
[113] Akimana C, Kway YA. *Francisella*-arthropod vector interaction and its role in patho-adaptation to infect mammals. Front Microbiol 2011;2:34.

1.4.1. Update on *Francisella tularensis* phylogeography

A study carried out by Sjödin et al. (2012) suggests that ancestral *Francisella* strains originated in marine habitats. According to these researchers, *Francisella* can be divided into two main genetic clades occupying both terrestrial and marine habitats. The first includes *F. tularensis*, *F. novicida*, *F. hispaniensis* and *Wolbachia persica*, and the second *F. philomiragia* and *F. noatunensis*. *F. philomiragia* is an opportunistic pathogen of humans associated to sea water and *F. noatunensis* is an important pathogen of farmed fish. In 2009, *F. cantonensis* was reported as a new species of the genus *Francisella* (Qu et al., 2009) and other studies have also identified several forms of *Francisella* from soil and water (Barns et al., 2005)(Petersen et al., 2009) and tick *Francisella*-like endosymbionts (FLEs) (Liu et al., 2016). In brief, the *Francisella* genus is expanding rapidly and its members appear to be associated with different ecological niches, ranging from specialised pathogens and endosymbionts with different host spectra, to generalists believed capable of a free-living existence (Sjödin et al., 2012).

F. tularensis genome displays very low variability (Kugeler et al., 2009). Nevertheless, *F. tularensis* subsp. *tularensis* (Type A) has been subdivided into 2 groups, A.I and A.II (Johansson et al., 2004)(Farlow et al., 2005)(Vogler et al., 2009). While group A.II is found mainly in the western United States (Farlow et al., 2005)(Staples et al., 2006) the group A.I is found throughout the central and eastern regions of the country and only sporadically in some western states (Farlow et al., 2005)(Staples et al., 2006)(Keim et al., 2007). Kugeler et al. (2009) identified four Type A strains clades, namely AIa, AIb, AIIa, and AIIb, in the United States using pulsed-field gel electrophoresis (PFGE). However, a more recent study using whole-genome sequencing (WGS), with higher phylogenetic resolution, displayed three major subgroups, A.I.3, A.I.8, and A.I.12, within group A.I, which names were assigned in consonance with previous phylogenetic nomenclature within *F. tularensis* by Gyuranecz et al. (2012) (Birdsell et al., 2014). This study revealed that all strains previously assigned to PFGE subgroup A1a belonged to the A.I.12 subgroup while the previous PFGE subgroup A1b strains were distributed among all three of the new subgroups, A.I.3 A.I.8 and A.I.12 (Birdsell et al., 2014). Using 16 canonical single nucleotide polymorphisms (canSNP) assays developed for the purpose, it was possible to further characterize each one of the three new subgroups. Hence, within the A.I.12 subgroup six subpopulations were identified occurring throughout the United States. Within group A.I.8 four subpopulations were found mostly occurring in the central and western regions of the United States, with only one strain isolated in the eastern region (**Figure 15 A and B**). Within subgroup A.I.3., four subpopulations were also identified,

showing a dramatically different distribution from the other subgroups (Birdsell et al., 2014). For the latter group, most strains were found to occur in the eastern region and just one subpopulation in the central region but none in the western region **Figure 15 A and B**) (Birdsell et al., 2014).

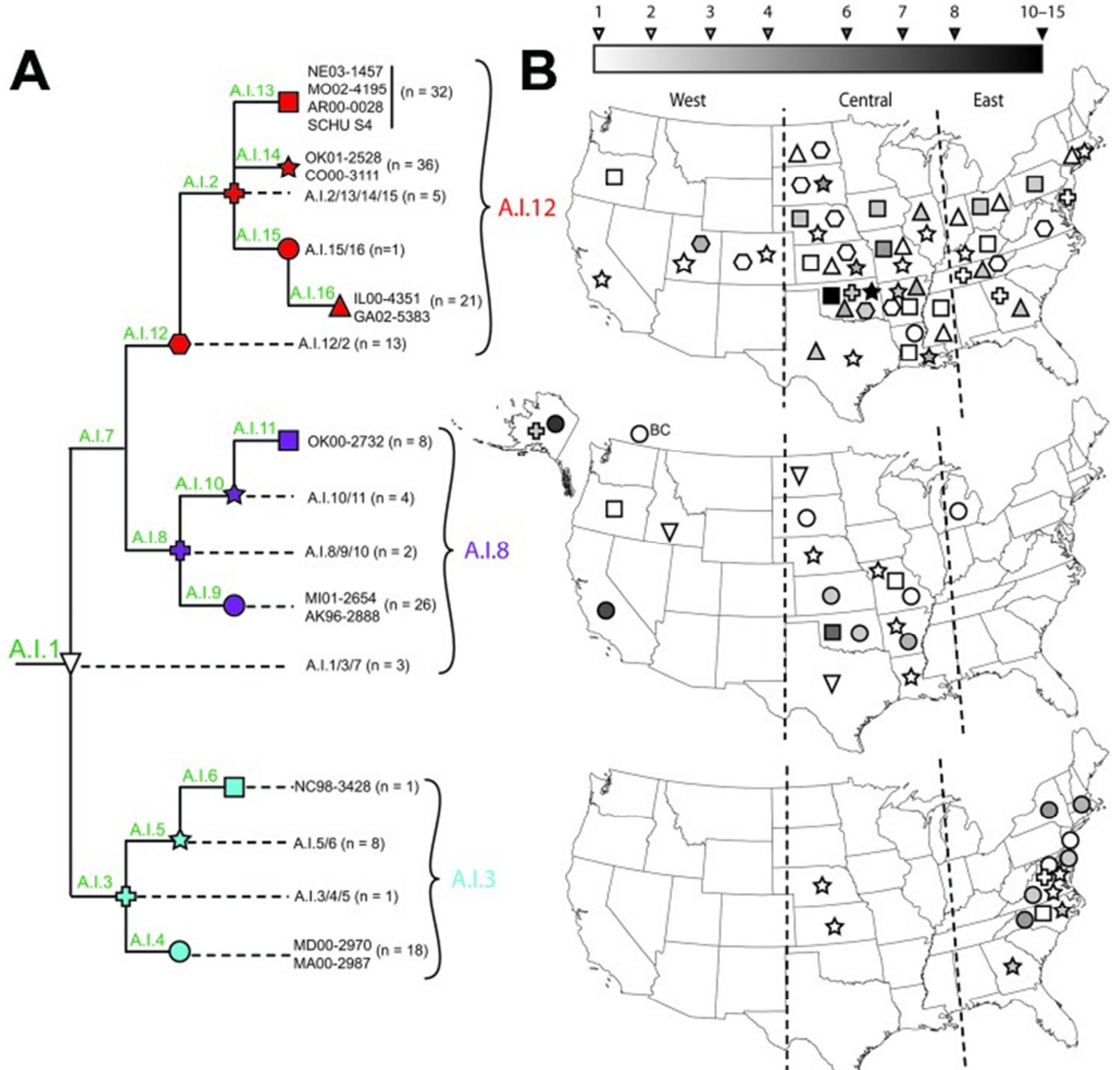


Figure 15. Phylogeography *Francisella tularensis* subsp. *tularensis* A.I strains from the United States, as obtained by (Birdsell et al., 2014). **(A)-** Canonical single nucleotide polymorphism (canSNP) of subpopulations. Colours indicate the major subgroups within A.I: A.I.12 is marked in red, A.I.8 in purple and A.I.3 in blue. Subpopulations are indicated by symbols and "n" refers to the number of strains. **(B)-** Geographic distribution of strains from the subpopulations shown in panel A, by corresponding symbols; top: A.I.12; middle: A.I.8 and bottom: A.I.3. Vertical lines indicate boundaries of the western, central, and eastern regions. Subgroups are mapped based on the geographic origin (state). Darker symbols indicate a higher number of strains; (adapted from (Birdsell et al., 2014)).

The results obtained by the Birdsell et al. (2014) phylogenetic study suggest a recent radiation evolutionary process for the A.I group (pattern of several short branches without hierarchical structuring), most likely in response to adaptive change, new ecologic opportunities, or a combination of these factors. If, in a phylogenetic context, the greatest genetic diversity implies ancient origins, Birdsell et al. (2014) results suggest the central United States as the most likely geographic origin of a common ancestor to *F. tularensis* Type A subgroups A.I.12 and A.I.8 and, perhaps, of the A.I group as a whole. The wide geographic range of the A.I.12 subgroup and the phylogenetic pattern of a long branch leading to a polytomy with genetic homogeneity, suggest a possible adaptive advantage for this subgroup, which might be related to differences in virulence among A.I strains.

The wide geographic success of *F. tularensis* is predominantly due to the spread of *F. tularensis* subsp. *holarctica* (Type B), but very little genetic diversity has been identified within this subspecies (Vogler et al., 2009). This lack of diversity, combined with the subspecies wide geographic distribution, may suggest that *F. tularensis holarctica* experienced recently a genetic bottleneck and expanded across the northern hemisphere (Dempsey et al., 2006)(Farlow et al., 2005)(Johansson et al., 2004)(Keim et al., 2007). Dwibedi et al. (2016) identified slow, but variable, replication rates for *F. tularensis* subsp. *holarctica* corresponding to null mutation rates based on the year of isolation and only in outbreak hotspots there was a rate of 0.4 mutations/ genome/ year. As a consequence, defining the population structure within this subspecies has been especially difficult (Vogler et al., 2009). All evidences point to a recent global expansion of *F. tularensis* subsp. *holarctica* and a radiation event (the B radiation) wherein this subspecies spread throughout the northern hemisphere (Vogler et al., 2009). Hypothesis on the geographic origin of *F. tularensis* subsp. *holarctica* suggest that the pathogen may have originated in Asia or, alternatively, in North America (Vogler et al., 2009).

In Europe, it is proposed that the Western regions were colonized by a monophyletic population of *F. tularensis* subsp. *holarctica* through the rapid clonal expansion of a specific population. As the east strains were more diverse, it is suggested that the founder population originated here and the other regions have been colonized by clonal descendants (Dwibedi et al., 2016). This is in agreement with the epidemiology records of the first tularaemia outbreaks in south-western areas of Spain as recently as in the 90's (Gutiérrez et al., 2003).

The diversity *F. tularensis* subsp. *holarctica* in Europe seems to be influenced by micro-evolution with limited dispersal (identical or genetically very closely related strains from small geographic

areas) wherein bacteria are accumulating genetic diversity and expanding, and by very long-distance and rapid movements of recent bacterial ancestors, with a very low degree of dispersal limitation (genetic diversity imports and highly similar genotypes at large distances). A weak correlation between genetic distance and geographic distance was found (Dwibedi et al., 2016).

Aside from mutations rates lower than one nucleotide substitution every second year per genome, Dwibedi et al. (2016) also found patterns of nucleotide substitution showing marked AT mutational bias suggestive of genetic drift. It seems also that *F. tularensis* subsp. *holarctica* is able to survive long periods inactive and with little replication between epidemics (Johansson et al., 2014), *i.e.* a resting phase for long-term survival (Romanova et al., 2000).

In brief, recent data indicate a founder population spreading in big jumps from east to west in Europe, and that the genetic diversity within this subspecies seems to have been generated by the interaction of mutation-driven evolution, a resting survival phase, genetic drift and long-distance geographical dispersal events (Dwibedi et al., 2016).

In Europe, two main genetic groups of *F. tularensis* subsp. *holarctica* were differentiated by high resolution molecular techniques (WGS with sSNP), the B.12/B.13 and B.FTNF002-00 which showed distinct geographic distribution (Vogler et al., 2009). The B.FTNF002-00 group was found to be dominant in Western European countries (France, Italy, the Netherlands and Spain) while the B.12/B.13 group was isolated mainly in Northern, Central and Eastern Europe (Austria, Czech Republic, Finland, Hungary, Norway, Romania, Slovakia, Sweden, Ukraine) and in the European part of Russia (**Figure 16**) (Kreizinger et al., 2016). Both genotypes were detected in Germany and Switzerland (Vogler et al., 2009)(Gyuranecz et al., 2012).

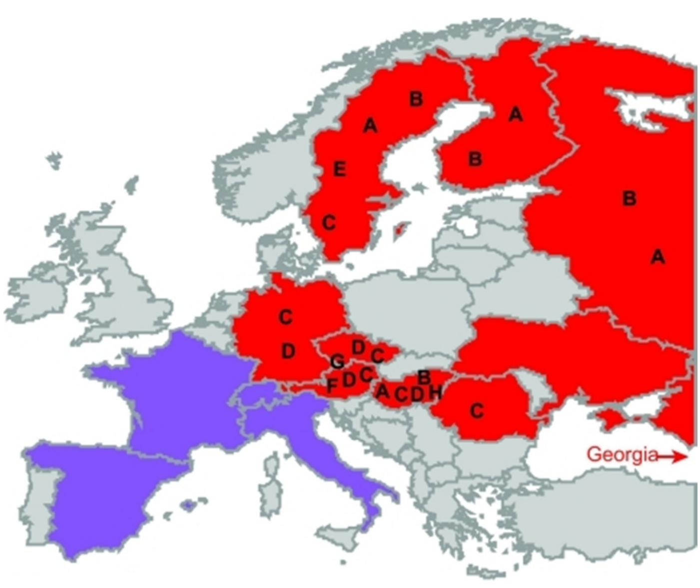


Figure 16. Geographic distribution of *Francisella tularensis* subsp. *holarctica* groups B.13 (red) and B.FTNF002–00 (purple) (adapted from (Gyuranecz et al., 2012)).

Whole genome analysis of B.FTNF002-00 and B.12/B.13 genotypes revealed distinct genetic differences, namely smaller genome size of genotype B.FTNF002-00, differences in gene sizes or orientations and the effects of SNPs in protein coding, which may explain the enhanced virulence and replication potential of this genotype (Keizinger et al., 2016).

Francisella tularensis subsp. *holarctica* strains are classically differentiated into three biovars (Olsufjev et al., 1983): a) Biovar I found in Western Europe and North America; b) Biovar II found in Eastern Europe and Asia (Vogler et al., 2009)(Gyuranecz et al., 2012); and c) Biovar japonica mainly found in Japan, although recently described in China (Wang et al., 2014) and Turkey (Kiliç et al., 2013). Both biovar I and II strains coexist in Germany, Switzerland and Scandinavia (Müller et al., 2013)(Origgi et al., 2014)(Maurin and Gyuranecz, 2016). The four main clades of *F. tularensis* subsp. *holarctica* include clade B.4 corresponding to North American strains, clade B.6 corresponding to biovar I Western European strains, clade B.12 corresponding to biovar II Eastern European strains, and clade B16 corresponding to strains belonging to biovar japonica (Fujita et al., 2008)(Vogler et al., 2009)(Karlsson et al., 2016)(Caspar and Maurin, 2017).

1.4.1.1 Update on *Francisella*-like endosymbionts

This information is given in chapter VIII.

1.4.2. Update on *Francisella tularensis* epidemiology

This information is given in chapter VII.

1.4.3. Update on *Francisella tularensis* life cycle

Regarding the role of free-living amoeba (FLA) in the *F. tularensis* life cycle, a recent study showed that *Francisella* spp. survival was enhanced by the presence of FLA although bacterial growth and protozoa infectivity were not observed. Although short-term incubation with FLA was shown to be beneficial, the long-term effects on *Francisella* survival are yet unknown, (Buse et al., 2017).

1.4.4. Update on *Francisella tularensis* immunopathogenesis

The virulence of *Francisella tularensis* relies on an atypical type VI secretion system (T6SS) encoded by the genomic "*Francisella* Pathogenicity Island" (FPI) (Rigard et al., 2016), highly conserved between *F.tularensis* and *F.novicida* (>97% identity at the nucleotide level (Rigard et al., 2016). For the latter, this genomic island is called the "*Francisella novicida* Island" (FNI) and must likely encode another atypical T6SS (Rigard et al., 2016).

Alongside with the macrophage receptors involved in *Francisella* uptake addressed in General Introduction, the scavenger receptor A (SR-A) (Geier and Celli, 2011), Fcγ receptors (Balagopal et al., 2006), nucleolin (Barel et al., 2008), and the lung surfactant protein A (SP-A) (Balagopal et al. 2006) are also thought to be implicated in the uptake of serum-opsonized *Francisella* by macrophages (Celli and Zahrt, 2013). Following uptake, the pathogen resides within the "*Francisella*-containing phagosome" (FCP), a primary vacuolar compartment that undergoes progressive maturation into a bactericidal phagolysosome (Jones et al., 2011)(Celli and Zhart, 2013). The FCP acquires markers of early endosomal antigen 1 (EEA1) and late endosomal markers Lamp1, Lamp2, and the Rab7 GTPase within 15–30 min (Clemens et al., 2004)(Santic et al., 2005) (Checroun et al., 2006) indicative of a normal maturation process (Jones et al., 2011)(Celli and Zhart, 2013). Another feature of phagosomal maturation is the progressive acidification of the phagosomal lumen that acts as a signal for *F. tularensis* to escape from FCP (Celli and Zhart, 2013). The FCP does not fuse with lysosomes and bacteria physically disrupt the phagosomal membrane and escape into the host-cell cytosol (Jones et al., 2011)(Celli and Zhart, 201).

The FIP virulence genes, particularly genes comprising the *iglABCD* operon have been directly implicated in the escape of *F. tularensis* from the phagosome into the host cytosol and in the

inhibition of macrophage pro-inflammatory response (Rigard et al., 2016). However, it is unknown which FPI genes encode for structural component of the T6SS machinery and if the FPI secreted proteins are effector proteins (Rigard et al., 2016). Many of the FPI proteins are secreted into the macrophage cytosol (Ozanic et al., 2016) and recently the IglG protein (a metal-binding protein) was shown to be required for the pathogen escape into the host cytosol, triggering of the cytosolic innate immune responses and replication within macrophages (Rigard et al., 2016). IglG was demonstrated to possess two important domains, one conserved in more than 250 bacterial species (DUF4280, a PAAR-like domain) and one specific for the *Francisella* genus (directly involved in forming a protein complex with another virulence effector protein, the FPI-encoded IglF protein) (Rigard et al., 2016). In addition, also the lipoprotein IglE secreted in a T6SS-dependent manner was shown to be important in *F. tularensis* pathogenicity in the inflammasome activation and escape from phagosome (Bröms et al., 2016). Other determinants besides the FPI have also been implicated in the *Francisella* phagosomal escape such as the acid phosphatases of *F. novicida* (AcpA, AcpB, AcpC, and Hap) (Mohapatra et al., 2008), pyrimidine biosynthetic genes (carA, carB, and pyrB) (Schulert et al., 2009), as well as several genes of unknown function including *FTT1103* (Qin and Mann, 2006)(Qin et al., 2009) and *FTT1676* (Wehrly et al., 2009).

After *F. tularensis* escapes the FCP it replicates within the host-cell cytosol. A few genes have been identified as being specifically required for cytosolic replication by *Francisella* including the purine biosynthetic genes (purMCD) (Pechous et al., 2006)(Pechous et al., 2008), a g-glutamyl transpeptidase (ggt) (Alkhuder et al., 2009), and several genes of unknown function including *FTT0369c/ dipA* (Wehrly et al. 2009)(Chong et al., 2012), *FTT0989* (Brotcke et al., 2006), and *rip*A (Fuller et al., 2008). Yet, it is most likely that several additional genes may also contribute to this process (reviewed in (Chong et al. 2008)). Recent data showed that *Francisella* have also evolved mechanisms to adapt to a cytosolic lifestyle and to the host cytosol nutritional environment (Ziveri et al., 2017). This adaptation is associated with evolutionary loss of genes for many metabolic pathways (Rohmer et al., 2006)(Larsson et al., 2009), but *F. tularensis* has retained or evolved mechanisms to efficiently acquire essential nutrients from the host cytosol, mainly relying on host amino acid as major gluconeogenic substrates (Ziveri et al., 2017). For this purpose, the bacterium possesses high affinity uptake systems including amino acid transporters (the amino acid-polyamine-organocation transporters) the proton-dependent oligopeptide transporters (POT), the hydroxy/aromatic amino acid permeases (HAAAP) and the major facilitator superfamily (MFS) proteins involved in several transport functions, including amino acid uptake (Ziveri et al., 2017). In

addition, *Francisella* infection was shown to modify several "glyco-genes" involved in glycosylation pathways in human macrophages (Barel and Charbit, 2017). Iron, essential for key enzymatic and redox reactions, is also among the nutrients required to support The *F. tularensis* lifestyle and the bacterium relies on specialized mechanisms to acquire iron within the host environment (Ramakrishnan, 2017). The *F. tularensis* genome contains 5 homologous genes, *FTT0025c*, *FTT0267*, *FTT0602c*, *FTT0918*, and *FTT0919* that share 40–50% sequence identity (Larsson et al., 2005). Of these, *FTT0025c* (*FslE*) and *FTT0918* (*FupA*) have been shown to be involved in iron acquisition (Ramakrishnan et al., 2008)(Lindgren et al., 2009)(Sen et al., 2010)(Ramakrishnan et al., 2012), despite the iron acquisition systems of *F. tularensis* Type A and Type B strains appear to function distinctly (Wu et al., 2016).

Once *Francisella* is released in the macrophage cytosol is recognized in a type I IFN-dependent manner (Henry et al., 2007). *F. tularensis* lyse in the cytosol releases bacterial DNA that is recognized by the absent in melanoma 2 (AIM2) protein which in turn recruits ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain) and procaspase-1 to form an inflammasome complex (Jones et al. 2011). ASC is required to induce auto-proteolysis of pro-caspase-1, activating caspase-1. Active capsase-1 processes both pro-IL-1β and pro-IL-18 into their mature forms and triggers pyroptosis (host cell death) (Henry et al., 2007)(Jones et al., 2011). Recent data suggest that, besides caspase-1 and pro-inflammatory cytokines, the host guanylate-binding proteins (GBPs) also lead to the activation of the AIM2 inflammasome by triggering cytosolic bacteriolysis and might be involved in pyroptotic cell death (Meunier et al., 2015)(Wallet et al., 2016)Ziveri et al., 2017). In addition, a cell-to-cell transfer mechanism was recently described by Steele et al. (2016) for *Francisella*, suggesting that this pathogen could spread directly from infected to uninfected cells by trogocytosis. This is a pathogen driven process by which effector proteins nucleate and polymerize host cell actin physically propelling the bacteria into a neighbouring cell (Steele et al., 2016).

1.4.5. Clinical manifestations of tularaemia

Francisella infection outcomes are different depending on the route of infection, the first cells infected and on the immune response. For instance, alveolar macrophages are the first cells infected by the intranasal (i.n.) route, while neutrophils are the primary cells infected by the intradermal (i.d.) (Powell and Frelinger, 2017).

1.4.5.1. Humans

A recent study on the variation of the clinical manifestations of tularaemia carried out in Arkansas by Rothfeldt et al. (2017) from 2009 to 2013 (when this state had the highest tularaemia incidence on the United States (CDC, https://www.cdc.gov/tularemia/statistics/state), showed that lymphadenopathy was not the most common sign observed in that area. Although the majority of patients reported arthropod bites, infection eventually spread haematogenously without local signs in many cases and patients were more likely to report only generalized typhoidal symptoms. The typhoidal form of tularaemia can mimic other diseases in endemic regions, namely spotted fever rickettsiosis or ehrlichiosis, and might not be considered by clinicians until classic symptoms appear (Ellis et al., 2002)(Kugeler et al., 2009). *F. tularensis* Type A, prevalent in Arkansas and surrounding states (Ellis et al., 2002)(Kugeler et al., 2009), is more commonly associated with typhoidal presentation, especially in immunocompromised patients. Rothfeldt et al. (2017) study indicates that expecting lymphadenopathy as a primary symptom to screen for tularaemia may be an insensitive diagnostic strategy and delay case or outbreaks recognition. In addition it can also delay the appropriate case management and the presumptive treatment of patients during earlier infection stages, before serious disease. The medical community should be aware of the wide tularaemia presentations, particularly in endemic regions and in patients lacking local symptoms (Rothfeldt et al., 2017).

1.4.5.2. Animals

In the European brown hare (*Lepus europaeus*), the reservoir species of the bacterium in Central Europe, the clinical course of tularaemia was shown to have distinct patterns according to the geographical area and are directly related to the two predominant genotypes, the B.FTNF002-00 and B.12/B.13. While an acute course of the disease is observed in hares in Western European countries, where the B.FTNF002-00 group is dominant, sub-acute or chronic infections are more frequent in the eastern part as well as in North and Central parts of the continent, where B.12/B.13 is the predominant group (Kreizinger et al., 2016). In fact, the *F. tularensis* subsp. *holarctica* genotype B.FTNF002-00 higher pathogenicity in relation to B.12/B.13 genotype has been suggested (Origgi and Pilo, 2016). In Eastern Europe, including Hungary, necropsy findings in hares typically include granulomatous lesions in the lungs, pericardium and kidneys, due to a sub-acute or chronic infection (Gyuranecz et al., 2010). In contrast, signs of an acute clinical course of the disease, including splenomegaly, congestion and haemorrhagic lesions, have been described in

necropsies of hares originating from Western Europe, namely in France and in the Netherlands (Decors et al., 2011)(Rijks et al., 2013). Even in geographic regions were both genotypes are present, namely in Switzerland, lesions observed in brown hares infected by genotype B.12/B.13 differ from those of the genotype B.FTNF002-00 (Origgi and Pilo, 2016).

Recently, the natural history of pneumonic tularaemia in inbred Fischer 344 rat was reported by Hutt et al. (2017) and, overall, its pathogenesis appears to replicate the disease in humans. Female Fischer 344 rats were exposed to lethal doses of *F. tularensis subsp. tularensis* strain SCHU S4. Two days after exposure bacteraemia with haematogenous dissemination was detected. Shortly afterwards, the infected rats exhibited fever, tachypnea, and hypertension that persisted for 24 to 36 hours and then rapidly decreased as animals succumbed between days 5 and 8 after exposure. Tachycardia was observed briefly near death. Initial neutrophilic and histiocytic inflammation in affected tissues became progressively more fibrinous and necrotizing over time. At death, as many as 10 CFU were found in the lungs, spleen, and liver and death was attributed to sepsis and disseminated intravascular coagulation (DIC).

1.4.6. Update on the *Francisella tularensis* laboratorial diagnosis and genotyping methods

This information is given in chapter VI.

1.4.7. Update on tularaemia treatment

Although antibiotic susceptibility testing (AST) of *F. tularensis* strains is important for detection of antibiotic resistances to first-line drugs, as well as to test new therapeutic alternatives, it is rarely performed on a routine basis (Caspar and Maurin, 2017). This is because bacteriological isolation from blood or tissue samples is only obtained in <20% of patients (Maurin et al., 2011). In addition, this procedure may have risks for the laboratory personnel as it requires biosafety level 3 (BSL3) facilities (Tärnvik, 2007). Furthermore, acquired resistance to antibiotics has not been reported in clinical isolates of *F. tularensis* (Tärnvik, 2007).

Fluoroquinolones were considered the best choice for the treatment of uncomplicated tularaemia and aminoglycosides are generally used in most serious cases (reviewed in (Carvalho et al., 2014)). Novel antibiotics that can constitute potential options in the treatment of tularaemia include Azithromycin (an azalide) and telithromycin (the first ketolide antibiotic and a semi-synthetic

erythromycin derivative). Although confirmation of their efficacy in animal models is still lacking, there are evidences that they may be useful alternatives for infection caused by *F. tularensis* subsp. *holarctica* biovar I strains, when all first line antibiotics are contraindicated (Caspar and Maurin, 2017).

Rifampicin may be a good antibiotic choice in association with fluoroquinolones for rare bone and joint infections. Although this antibiotic is effective against tularaemia *in vitro* it should be further evaluated in animal models (Caspar and Maurin, 2017).

Among the antibiotics more recently developed, tigecycline has been evaluated for treatment of tularaemia. It is a new glycylcycline, a new class of antibiotics derived from tetracycline, that showed efficacy against tularaemia *in vitro* (Yesilyurt et al., 2011)(Kreizinger et al., 2013b). This new antibiotic might be a suitable alternative to doxycycline but its broader antibacterial spectrum can cause deleterious effects on the gut commensal flora (Caspar and Maurin, 2017).

Linezolid, an oxazolidinone compound, showed conflicting results, namely MIC variations between strains, when evaluated for treatment of tularaemia caused by Biovar II Type B. Differences were also observed between the susceptibility of Type A and Type B strains from the USA (Caspar and Maurin, 2017). The relative susceptibility of *F. tularensis* to linezolid may be related to its small genome with a limited number of efflux systems, and MIC variations between strains may reflect the variable expression of these efflux systems (Caspar and Maurin, 2017).

TP-271 is a novel, fully synthetic fluorocycline that is being evaluated for complicated bacterial respiratory infections (Grossman et al., 2017). According to Grossman et al. (2017), TP-271 showed to be active *in vitro* against *Francisella tularensis*. When tested in vivo for aerosolized exposure to *F. tularensis* in mice high survival rates (80% to 100%) were obtained and the surviving mice showed little to no relapse during 14-days post-treatment. In a non-human primate model (cynomolgus macaques) of inhalational tularaemia, all animals treated with TP-271 survived until the end of the study, with no relapse during 14 days post-last treatment. The protection and low relapse afforded by TP-271 treatment in supports a continued investigation of TP-271 effort in the event of aerosolized exposure.

1.4.8. Update on tularaemia prophylaxis

Francisella tularensis is 'Category A' agent and, in a bioterrorism scenario, vaccines and therapeutics are immediately required (Gaur et al., 2017). Currently, there is no licensed vaccine for tularaemia in the USA since none of the vaccine prototypes for *Francisella* was approved by the

Food and Drug Administration (FDA) (Golovliov et al., 2016). As tularaemia is an unusual disease in most countries and, even in endemic areas, it appears irregularly (Sjöstedt, 2007), evaluation of vaccine efficacy is hampered (Golovliov et al., 2016).

A vaccine for tularaemia needs to be evaluated according to the FDA (Snoy, 2010) whose regulations stipulate that efficacy testing of vaccines against tularaemia can be performed using animal models (mouse, rat, rabbit and non-human primate models) (Golovliov et al., 2016) or, alternatively, using the splenocyte-BMDM (bone marrow-derived macrophages) co-culture model (Golovliov et al., 2016). Besides allowing for direct comparisons of correlates with those identified in human models, this model reduces the number of animals required while refining the protocol once the mice that are immunized as a source of splenocytes receive a sublethal dose with minimal distress and few or no objective symptoms (Golovliov et al., 2016).

Safe vaccine prototypes against tularaemia included attenuated mutant *Francisella*, virus vector, and subunit immunogens with carrier system (Gregory et al., 2009). A subunit vaccine using immunodominant antigens, including outer membrane proteins (OMPs), has also been considered (Huntley et al., 2008)(Ashtekar et al., 2012). OMPs are involved in various virulence processes, including protein secretion, host cell attachment, and intracellular survival (Wu et al., 2016). OMPs of *Francisella* strains, namely *fop*A and *tul*4, induce immunogenicity by eliciting specific antibodies and have been studied as strong vaccine candidates against tularaemia (Hickey et al., 2011)(Ashtekar et al., 2012). In addition, a combination of *fop*A and *tul*4 immunogenic epitopes elicited an initial immune response against tularaemia related with dendritic cells *in vitro* and humoral immunity *in vivo*, and could constitute a safe and effective potential vaccine candidate (Oh et al., 2016).

Roberts et al. (2017) identified an important role for high avidity CD4+ T cells in short-term protection and hypothesized that increasing this pool of cells could improve vaccine efficacy. They also showed that a prime/boost vaccination strategy increased the pool of high avidity CD4+ T cells, correlating with improved survival after challenge with high doses of virulent *F. tularensis* subsp. *tularensis* or at late time points after vaccination. The Roberts et al. (2017) results suggest that both epitope selection and vaccination strategies increasing antigen-specific T cells correlate with higher immunity against *F. tularensis* subsp. *tularensis*.

Also recently, an immunoproteomic approach based on the techniques of 2-dimensional gel electrophoresis (2DE) and immunoblotting combined with mass spectrometry (MS) was used for elucidation of immunogenic components and putative vaccine candidates for tularaemia. Eight new

immunogenic proteins were found in this study, which may be used in design and development of a protein subunit vaccine for this disease (Gaur et al., 2017).

Golovliov et al. (2017) analysed if specific mutants of SCHU S4 (Type A) could serve as vaccine candidates and, using the splenocyte-BMDM co-culture method, identified the 1clpB mutant which conferred superior efficacy (survival rates after infection with SCHU S4) when compared to LVS. These findings demonstrate a strong correlation between the ability of the vaccine strains to confer protection to virulent strains *in vivo* and their competence to efficiently prime the protective efficacy of the immune cells as measured by the splenocyte BMDM co-culture model.

Richard et al. (2014) demonstrated that, incorporating lysates from partially attenuated *F. tularensis* LVS or fully virulent *F. tularensis* SCHU S4 strains into catanionic surfactant vesicle (V) nanoparticles, fully protected mice against *F. tularensis* LVS (by the intraperitoneal route, i.p.) but conferred only partial protection against *F. tularensis* SCHU S4 (by intranasal route, i.n.) even when employing heterologous prime-boost immunization strategies. Later, the same researchers showed that both LVS-V and SCHU S4-V immunization inoculated via i.p. elicited similarly high titers of anti-*F. tularensis* IgG that could be further increased by adding monophosphoryl lipid A (MPL), a nontoxic Toll-like receptor 4 (TLR4) adjuvant that is included in several FDA-approved vaccines in the USA (Richard et al., 2017). Active immunization with LVS-V+MPL (i.p./i.p.) also increased the frequency of gamma interferon (IFN-γ)-secreting activated helper T cells, IFN-γ production, and the ability of splenocytes to control intra-macrophage infection. In addition, LVS-V+MPL immunization via heterologous routes (i.p. and i.n.) significantly elevated IgA and IgG levels in bronchoalveolar lavage fluid and enhanced protection (~60%) against intranasal challenge with *F. tularensis* SCHU S4. These data represent a significant step in the development of a subunit vaccine against the highly virulent Type A strains (Richard et al., 2017).

A recent study, carried out by Kumar et al. (2017) directly demonstrated that *F. tularensis* growth conditions strongly impact on vaccines and that the growth medium used to produce whole cell vaccines against *F. tularensis* is important in vaccine development. In fact, inactivated *F. tularensis* grown in Mueller Hinton Broth (MHB) exhibited superior protective activity when used as a vaccine, as compared to Brain-Heart Infusion (BHI).

2. Results obtained: how and in what way have these contributed to our knowledge of RHD and Tularaemia

Chapter I:

The RHD Laboratorial Diagnosis

1. A review on the RHD laboratorial diagnosis

Although the epidemiological features, clinical signs and pathological changes often allow the presumptive diagnosis of RHD (Capucci et al., 1991), several methods were developed for the RHDV laboratorial diagnosis since it was first detected in 1984 (Liu et al., 1984). Those methods are based on the direct observation of viral particles, but also on the detection of viral proteins, antigens, specific antibodies or viral nucleic acid (RNA).

1.1. Sample preparation

Laboratory diagnosis samples may include fresh liver, spleen and blood or formalin-fixed samples of liver, spleen, lung, kidney and other organs (OIE Technical Disease Cards, 2015 update). As the liver contains the highest viral titre (from 10^3 LD_{50} [50% lethal dose] to $10^{6.5}$ LD_{50}/ml of 10% homogenate), is the organ of choice for viral identification in acute or peracute disease. The quantity of virus present in other tissues is directly proportional to their vascularisation. Serum and spleen may also contain high titres of virus and, in rabbits with chronic or subacute disease, the spleen may be preferable over the liver for RHDV detection. Serum may serve as alternative diagnostic material (OIE Terrestrial Manual 2016).

Regardless of the diagnostic method used, and with the exception of immunostaining techniques, samples are treated equally (OIE Terrestrial Manual 2016). An organ fragment is mechanically homogenised in phosphate-buffered saline solution (PBS, pH 7.2) at 5-20% w/v and clarified by centrifugation at 3-8,000 rpm for 5-15 min (Capucci et al., 1991). At this stage, the supernatant can be directly submitted to the HA test or enzyme-linked immunosorbent assay (ELISA). A second centrifugation at 10-12,000 rpm for 5 to 15 min is advisable for electron microscopy, before the final ultracentrifugation (Capucci et al., 1991). For PCR detection, viral RNA may be directly extracted from tissues samples (OIE Terrestrial Manual 2016).

For RNA preservation it is preferable to carry out all preparation steps at 4°C (Capucci et al., 1991).

1.2. Detection Methods

1.2.1. Direct diagnostic methods

Direct methods include the observation of viral particles or the detection of viral antigen, protein or RNA.

1.2.1.1. Electron microscopy

Electron microscopic (EM) diagnosis is suited for rapid identification of infectious agents (Hazelton and Gelderblom, 2003) and is the only technique able to deliver clear images of viruses, due to their small size (Roingeard, 2008).

Negative staining uses heavy metals salts to provide contrast from viruses, which appear translucent in EM (Schramlová et al., 2010) and stand out from the background, providing morphological information on symmetry and capsomer arrangement (Roingeard, 2008). Phosphotungstic acid (PTA) is the most commonly used negative stain (Roingeard, 2008)(Schramlová et al., 2010). Suspensions of viruses must be supported on thin of plastic, carbon, or a combination of the two applied to the surface of an electron microscope grid (Hazelton and Gelderblom, 2003)(Schramlová, 2009).

For RHDV identification, negative-staining EM can be performed using the "drop method". However, due to the lower sensitivity of this method, ultraconcentration of the viral particles is advisable (OIE Terrestrial Manual 2016).

The detection and identification of RHDV can also be accomplished by immune electron microscopy (IEM) as the immunological reaction induces the clumping of viral particles in easily identified aggregates (Capucci et al., 1991)(OIE Technical Disease Cards, 2015 update). IEM employs monoclonal antibodies (MAbs) or rabbit hyperimmune serum anti-RHDV increasing detection sensitivity, being considered the best EM method for RHDV diagnosis by OIE (Lavazza et al., 2015b)(OIE Technical Disease Cards, 2015 update)(OIE Terrestrial Manual 2016). The IEM technique proved to be more sensitive and specific than haemagglutination (HA) and nearly equal to enzyme-linked immunosorbent assay (ELISA) (Capucci et al., 1991). Although time-consuming and costly, it is advisable for doubtful cases (Capucci et al., 1991)(OIE Technical Disease Cards, 2015 update).

Immunogold is an IEM technique that utilizes specific antibodies tagged with small particles of colloidal gold as electron dense marker (Schramlová et al., 2010), and is mainly used for research purposes (OIE Terrestrial Manual 2016)(Schramlová et al., 2010).

The first electron microscopy images of this virus in Portugal were obtained by the Instituto Nacional de Investigação Veterinária (INIAV) team, in collaboration with the Faculdade de Medicina Veterinária (FMV) (**Figure 17**).

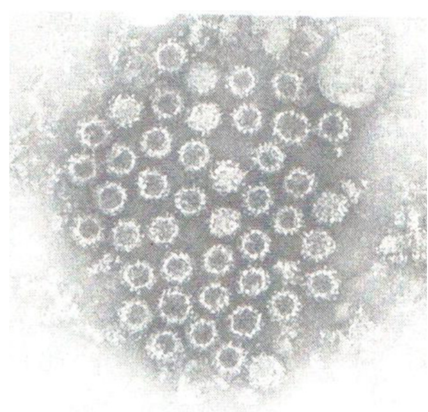


Figure 17. RHDV electron microscopy image. This was the first EM photo of RHDV obtained in the Instituto Nacional de Investigação Agrária e Veterinária (INIAV), in 1989. Photo kindly relinquished by Doutora Margarida Duarte (INIAV).

1.2.1.2. Antigen detection

1.2.1.2.1. Haemagglutination (HA) test

The haemagglutination (HA) test was the first method used for the laboratory diagnosis of RHD (Pu et al., 1985)(Capucci et al., 1991). This test is performed on 10% (w/v) tissue homogenate of liver or spleen (OIE Technical Disease Cards, 2015 update) or alternatively of lungs and kidneys (Capucci et al., 1991).

Liu et al. (1984) referred RHDV haemagglutinating properties towards human erythrocytes type "O" when discussing the first outbreaks. The RHDV ability to strongly agglutinate human red blood cells (RBC), constitutes an important feature of RHDV that was explored for specific diagnosis and viral detection in tissues of victimized rabbits (Ohlinger et al., 1990)(Capucci et al., 1991)(Chasey, 1997).

Haemagglutination titres have been detected in liver tissue of infected rabbits as early as 2h post infection and observed to rise rapidly, reaching a maximum at death (as high as 10×2^{14} to 10×2^{16}) (Ahmad et al., 2011)(Yang et al., 1989a)(Yang et al., 1989b). Agglutination at an end-point dilution greater than 1/160 is considered positive (Ahamad et al., 2011).

The HA is less sensitive and specific than other assays (Capucci et al., 1991) and not satisfactory for RHDV diagnosis when used alone (Chasey et al., 1995). Occasionally, false negative results (around 10% of samples), occur in rabbits showing a subacute/chronic form of the disease (OIE Terrestrial Manual 2016) due to non-haemagglutinating isolates of the virus (Chasey et al., 1995). HA has also failed to detect infection whenever virus particles have undergone proteolytic degradation (Capucci et al., 1991), due to autolytic processes or to physiological alterations of the host. Likewise, false

positive results (Capucci et al., 1991) have been reported and positive results must be confirmed by other methods such ELISA, EM, or immunostaining (Capucci et al., 1991)(Ahmad et al., 2011).
Regarding RHDV2, HA assays using human O, A, B, and AB erythrocytes showed that this new virus (as well as RHDV G1 and G6 strains), interacts with the different blood types in various ways (Dalton et al., 2012)(Leuthold et al., 2015). Some authors have referred that RHDV2 agglutinates human type "O" erythrocytes efficiently, making HA suitable for RHDV2 diagnosis (Le Gall-Reculé et al., 2013), while others mentioned that RHDV2 displays a pattern similar to G4 and G6 groups with no agglutination of blood groups "O" or "A", but agglutinating blood groups B and AB (Dalton et al., 2012).
Given the practical difficulty of obtaining and keeping human RBC, the risks associated with these cells, and the difficulty of obtaining consistent results (Capucci et al., 1991)(OIE Terrestrial Manual 2016), the HA assay was replaced by virus detection ELISA due to its simple methodology, inherent high sensitivity, and adequateness for testing large numbers of samples (Capucci et al., 1991)(OIE Technical Disease Cards, 2015 update).

1.2.1.2.2. Monoclonal antibodies (MAb)-based ELISA

ELISA methods based on the sandwich technique were developed in Italy for the veterinary diagnosis of RHD in domestic rabbits (Capucci et al., 1991)(Capucci et al., 1995). These methods consist of coating assay plates, with MAbs (or alternatively with polyclonal antibodies) that recognize different epitopes on the RHD viral capsid (Capucci et al., 1991). MAb-based ELISAs resourcing to (50) different MAbs were developed by the OIE Reference Laboratory for RHD (IZSLER, Brescia, Italy) enabling the subtyping of classical RHDV isolates (OIE Technical Disease Cards, 2015 update).
The various ELISA reactions developed for RHDV antigen detection differed in the enzymatic system and in the immunological reagents used as "catcher" and "tracer". All included the direct adsorption of a rabbit hyperimmune anti-RHD serum on the solid phase (a microplate of high adsorption capability) but different "tracers" are used (Capucci et al., 1991). As MAbs recognising specific RHDVa and RHDV2 epitopes, that can be used instead of rabbit polyclonal sera, have also been produced (OIE Terrestrial Manual 2016)(Le Gall-Reculé et al., 2013), the OIE advises to test each sample in at least four replicates, and then use horseradish peroxidase (HRPO) conjugates with different specificity.

Samples are positive if showing a difference in absorbance >0.3 between the wells coated with RHDV-positive serum and wells coated with the negative serum. Usually, at the dilution 1/30, positive samples from rabbits with the classical acute form of RHD give an absorbance value >0.8, while the absorbance value of the negative sample, at the dilution 1/5, ranges from 0.1 to 0.25 (OIE Terrestrial Manual 2016).

ELISA has proved significantly more sensitive and specific than HA. In addition, the combined use of the monoclonal and polyclonal antibodies in ELISA also provides a differential diagnostic method capable of distinguishing RHDV from EBHSV (Capucci et al., 1991).

1.2.1.2.3 Antigen sandwich ELISA

An *in house* antigen ELISA was developed by José Dias Vigário in the 90's and used in Instituto Nacional de Investigação Agrária e Veterinária (INIAV) (former Laboratório Nacional de Investigação Veterinária, LNIV) for RHDV diagnosis, until the implementation of molecular methods in 2004 (Duarte M., *personal communication*).

Briefly, 96-well immune-plates were covered with anti-RHDV IgGs (prepared from a polyclonal serum) and incubated overnight at 4ºC. Positive and negative control sera, at a predetermined dilution, are added to the plate, to which serial dilutions of antigen-containing sample (usually 1:5 to 1:160) were immediately applied in duplicated. After incubation and washing an anti-RHDV specific antibody marked with biotin is added, and revealed with a peroxidase Streptavidin conjugate, previously titrated. Peroxidase activity for the organic substrate o-phenylenediamine (OPD) in the presence of hydrogen peroxide (H_2O_2) is measured at 492 nm after a final wash to remove the unbound antibody-enzyme conjugate to determine the presence of antigen (Duarte M., *personal communication*).

1.2.1.2.4. Commercial kits

For RHDV antigen detection, commercial test kits based on VLPs were developed (INGEZIM RHDV DAS (Ingenasa, Madrid, Spain) (Dalton et al., 2014)(Bárcena et al., 2015).

In 2015, a recombinant baculovirus expressing the RHDV2 VP60 protein was generated leading to the production of VLPs that may constitute an alternative method for antigen production, avoiding the manipulation of RHDV2-infected rabbit liver extracts (Bárcena et al., 2015).

1.2.1.3. Western blotting

Western blotting analysis enables the identification of specific proteins (Mahmood and Yang, 2012). For RHDV detection, it is useful when other tests such as HA or ELISA give doubtful results (low positivity) or the samples are suspected of containing s-RHDV particles (OIE Terrestrial Manual 2016). Homogenates are prepared as described above for ELISA techniques but virus particles are further concentrated by ultracentrifugation through a 20% (w/w) sucrose cushion. Both the supernatant and the pellet can be used to detect the RHDV subunits (Capucci et al., 1995) and the denatured VP60 protein or its proteolytic fragments (OIE Terrestrial Manual).

Sample proteins are denatured by heat in the presence sodium dodecyl sulphate (SDS), and beta-mercaptoethanol, separated by polyacrylamide gel electrophoresis (SDS/PAGE), and transferred by electroblotting into nitrocellulose or PVDF (polyvinylidene flouride) membranes (Capucci et al., 1995)(OIE Terrestrial Manual 2016). After transfer, the membranes are saturated and subsequently incubated with the appropriate serum dilution (Capucci et al., 1995)(OIE Terrestrial Manual 2016). The filters are washed and incubated with anti-species alkaline phosphatase-labelled immunoglobulins at a dilution predetermined by titration. Finally, chromogenic substrate (5-bromo-4-chloro-3-indolylphosphate nitro blue tetrazolium) is added (OIE Terrestrial Manual 2016). A positive and negative sample should be used as controls. Positive test samples and the positive control will produce a pattern consistent with reaction to proteins of molecular weights of, respectively, 60 kDa (the single structural protein of RHDV) or 41–28 kDa (the fragments of the VP60 associated with the transition from RHDV to s-RHDV), when examining the pellet, and 6 kDa (the subunits) when examining the supernatant.

RHDV proteins can be detected with polyclonal antibodies or MAbs. The later MAbs should recognise continuous epitopes (Capucci et al., 1995). Rabbit anti-RHDV hyperimmune sera are less efficient than MAbs at recognising the same band patterns (Capucci et al., 1996).

1.2.1.4 Molecular methods

1.2.1.4.1 Reverse transcriptase polymerase chain reaction (RT-PCR)

Because RHDV non-cultivable nature, quick diagnostics tests have relied greatly in serologic assays and molecular methods (reviewed by Niedzwiedzka-Rystwej et al., 2013). RT-PCR for RHDV detection can be performed on organ samples (optimally liver or spleen), urine, faeces and sera (OIE technical disease cards, 2015 update)(OIE Terestrial Manual 2016).

1.2.1.4.1.1. Conventional PCR

The first described RT-PCR method for RHDV detection was a conventional system (Guittré et al., 1995). This method amplified the N-terminal portion of the RHDV capsid protein encoding region and proved to be 10^4 fold more sensitive than ELISA testing for virus detection, while detecting as few as 12 copies of template cDNA (Guittré et al., 1995). The amplified DNA reaction mixture is subjected to electrophoresis on agarose gel. Specificity of the PCR product can be further confirmed by sequencing analysis. When combined with sequencing, the method demonstrated that the amplified portion of the RHDV capsid protein is highly conserved among RHDV isolates, even from geographically and temporally separate outbreaks (Guittré et al. 1995)(Guittré et al., 1996). Since then, other conventional RT-PCR assays were described (Ros Bascunana et al., 1997) Tham et al. 1999)(Yang et al., 2008).

The OIE Reference Laboratory for RHD uses a single-step RT-PCR, with the following *vp60* gene specific primers: forward primer 5′-CCT-GTT-ACCATC-ACC-ATG-CC-3′ and reverse primer 5′-CAA-GTT-CCA-RTG-SCT-GTT-GCA-3′ (OIE Terrestrial Manual 2016). The primers are able to amplify all RHDV genogroups and RHDV2. For the specific amplification of RHDV2, the primers are: "14U1" (5′-GAA-TGT-GCT-TGA-GTT-YTG-GTA-3′) and "RVP60-L1" (5′-CAA-GTCCCA-GTC-CRA-TRA-A-3′), amplifying a 794 bp sequence located in the C-terminal of the gene encoding the VP60 of RHDV2 (Le Gall-Reculé et al., 2013).

1.2.1.4.1.2. Immunocapture-RT-PCR

An immunocapture-RT-PCR method was developed by Le Gall-Reculé et al. (2001) for the detection and genomic characterization of both RHDV and EBHSV. The method was based on viral purification by immunocapture and genomic amplification by reverse transcription-polymerase chain reaction (IC-RT-PCR). It has the advantage to be applied directly to liver preparations obtained after thawing, suppressing the viral nucleic acid preparation step. The assay combines the rapidity of an ELISA test as immunocapture and the RT reaction are carried out in the same microtitre plate, with the sensitivity of PCR. Furthermore, it allows the processing of large numbers of samples and proved suitable for lagomorphs' caliciviruses phylogenetic studies (Le Gall-Reculé et al., 2001).

1.2.1.4.1.3. Real-time RT-PCR

The real-time RT-PCR method proved to be appropriate for assessment of liver samples from RHDV-positive rabbits (Niedźwiedzka-Rystwej et al., 2013). In real-time RT-PCR, the detection of specific gene sequences involves monitoring of the fluorescence generated by cleavage of a target specific oligonucleotide probe during amplification (Fitzner et al., 2011). The technique is highly sensitive and specific allowing the significant shortening of the reaction time, due to elimination of the post-amplification processing electrophoretic analysis phase. Also, real-time RT-PCR usually targets smaller regions.

Gall et al. (2007) developed a highly sensitive and specific real time multiplex RT-PCR for RHDV detection, appropriate for quantitative investigations of RHDV. This TaqMan Probe system was able to identify RHDV infection in convalescent rabbits. The systems' ability to detect viral RNA was measured at 10 copies per well, and linearity over a range from 10^1 to 10^{10} copies was demonstrated (Gall et al., 2007).

Fitzner et al. (2011) also developed a highly sensitive single one-step TaqMan real-time RT-PCR method for virological diagnosis of RHD. The limit of detection of viral RNA extracted from the liver of rabbits infected with known virus strains was established between 10^{-7} and 10^{-8} dilution tool (Fitzner et al., 2011).

More recently, loop-mediated isothermal amplification (LAMP) (Yuan et al., 2013) and SYBR green-based real-time PCR (Niedzwiedzka-Rystwej et al., 2013)(Liu et al., 2015) methods were also described. LAMP was developed by Notomi et al. (2000) allowing a rapid and highly specific amplification under isothermal conditions. This method employs a DNA polymerase and four specially designed primers that recognize six sequences on the target DNA. LAMP produces stem-loop DNA structures with various lengths that can be detected by a ladder pattern of bands on a DNA agarose gel or be visualized as precipitates in a turbid solution, with no specific reagent or equipment requirement (Notomi et al., 2000). SYBR green real-time PCR method is based upon the binding of the fluorescent dye SYBR-Green I into the PCR product (Ponchel et al., 2003). It is as rapid and sensitive as TaqMan, but less expensive (Ponchel et al., 2003).

Given that the molecular assays previously described are not suitable for RHDV2 detection, the rapid spread of the emerging RHDV2, with dramatic impacts on the wild rabbit populations and rabbit industry, prompted us to develop a specific and fast laboratorial diagnostic method for RHDV2 detection (Duarte el al. 2015). This molecular tool is particularly important to assist the veterinarians in charge of the control of the infection in rabbit industries (Duarte et al., 2015). This

RHDV2 specific Taqman-probe-based real time PCR (RT-qPCR) provides a clear diagnosis in less than 3 hours. To the best of our knowledge, it is still the only available method and figures in the World Organization for Animal Health (OIE) Terrestrial Manual from 2016. The method showed high sensitivity, detecting as few as 9 copies of RDHV2, allowing the measurement of RHDV2 viral loads. The method was tested in parallel with other methods between 2014 and 2015 before being published. The development and validation of this specific real-time for RHDV2 diagnosis was published in the *Journal of Virological Methods*, and is addressed in **Study 2** of this chapter. In addition, the challenges that the molecular diagnosis of RHDV2 may face, namely when applied to vaccinated animals, are addressed in **Study 3**.

1.2.1.5. Immunostaining

Immunostaining techniques can be used for RHDV detection by means of an Avidin-Biotin-Complex (ABC)-Peroxidase method on paraffin embedded formalin fixed tissue sections (Stoercklé-Berger et al., 1992). The sections are first deparaffinised in xylene and alcohol, counter-stained with haematoxylin and eosin. The tissue sections are then transferred to a methanol bath containing 3% H_2O_2 and washed in PBS. The samples are incubated with normal rabbit serum prior to the addition of biotin, to limit background interference caused by nonspecific antibody binding. Samples are then incubated overnight with biotinylated rabbit anti-RHDV serum or MAbs, washed and incubated with an ABC peroxidase, after which they are washed again. Amino-ethyl-carbazole is used as substrate. Finally, the slides are rinsed in tap water and mounted (Stoercklé-Berger et al., 1992). The intense nuclear and the diffuse cytoplasmic staining of necrotic hepatocytes, mainly in the periportal areas, are characteristic and specific. Positive staining of macrophages, Kupffer cells and hepatocellular reactions can also be observed. Likewise, positive reactions can be detected in lung, spleen and lymph nodes macrophages, and in the renal mesangial cells (Stoercklé-Berger et al., 1992).

The immunostaining technique can be applied to tissue cryosections fixed in methanol or acetone, incubated with fluorescein-conjugated rabbit anti-RHDV serum or MAbs. Specific fluorescence can be detected in the liver, spleen, and renal glomeruli (Gregg et al.,1991)(OIE Terrestrial Manual 2016).

1.2.1.5.1. Immunohistochemical techniques

Although less important in routine diagnosis, immunohistochemical procedures have revealed the presence of RHDV2 viral particles in the liver and in the small intestine (Dalton et al., 2012), similarly to what was observed for RHDV (Abrantes et al., 2014),.

Antigen for RHDV was detected in degenerative and necrotic hepatocytes, in areas of the histopathological lesions. The antigen (stained deep brown) was expressed in the cytoplasm (diffusely or concentrate as fine granules) of the hepatocytes, suggesting that RHDV replicated in these cells, and also observed in the spleen (Park and Itakura, 1995).

This technique detected RHDV viral antigen in macrophages, circulating monocytes and reticulo-endothelial cells of the liver, lung, spleen and lymph nodes, using antisera from naturally infected animals. In addition, at early infection, the RHDV VP60 protein was detected by immunohistological localization in tissues from experimentally infected adult and young rabbits, using a guinea pig polyclonal antibody raised against the recombinant RHDV VP60 protein (Prieto et al., 2000).

1.2.1.6. *"In-situ"* hybridisation (ISH) technique

An "*in-situ"* hybridisation (ISH) technique for the detection of rabbit haemorrhagic disease virus (RHDV) was developed in the late 90's (Gelmetti et al., 1998). The system described the use two RNA probes (sense and antisense) transcribed *in vitro* and UTP-digoxigenin-labelled (Gelmetti et al., 1998). This technique is highly sensitive and can detect RHDV as early as 6–8 hours after infection, but is mainly used in research (Gelmetti et al., 1998)(OIE technical disease cards, 2015 update). Non-isotopic "*in-situ"* hybridization proved suitable to study the RHDV distribution in tissues of infected rabbits (Kimura et al., 2001).

1.2.1.7. Viral isolation

Viruses can reach high titres when grown within susceptible cells (Leland and Ginocchio, 2007). Not all cell types support the replication of a given virus in relation to its host range and tissue tropism. The species from which the cell is derived, the lineage of the cell, and the degree of differentiation can have determinate whether a particular cell line will support the replication of a particular virus (Olivo et al., 1996).

Viruses' characteristics, their nucleic acid (DNA or RNA) content, capsid symmetry (icosahedral, helical, or complex), and the presence or absence of a lipid envelope (enveloped or naked), result in specific replication strategies. Viruses are obligate intracellular pathogens that rely on the cellular

machinery for all stages of the life cycle (Taube et al., 2010). The replication cycle of most viruses occurs as a result of complex interactions between virion-associated factors, virus-encoded factors, and host cell factors (Olivo et al., 1996). In most cases, viruses fail to replicate in a certain cell type by its inability to enter the cell, to express its genes or replicate its genome once inside the cell (Norkin et al., 1995). The absence of the appropriate virus receptor is probably the most common reason that obstructs virus entry into a cell (Norkin et al., 1995). In some cases, the receptor also plays an important role in entry *per se* in addition to its role in virus binding. The expression of the receptor on specific host cells or tissues is a major determinant of the route of virus entry, pattern of virus spread in the host and in pathogenesis (Norkin et al., 1995).

Viruses can bind many different molecules, typically glycoconjugates (glycosphingolipids, glycoproteins, and proteoglycans) (Taube et al., 2010). Some are attachment factors, concentrating virus on the cell surface, while others are receptors or co-receptors facilitating virus entry into cells. Viruses can also use alternate receptors depending on the cell type.

Since 1984, a number of attempts have been made to cultivate RHDV in various cell culture systems (reviewed in (Ahmad et al., 2011)). Primary rabbit cells (kidney, liver, lung and testis) as well as cell lines (PK-15, BHK-21, MA-104, IBRS-2, HeLa and VERO) have been used for the adaptation of RHDV to cell cultures, however, unsuccessfully (Ahmad et al., 2011).

For RHDV, rabbit inoculation remains the only way of isolating, propagating and titrating the virus infectivity. It should be considered only when inconclusive results are obtained by other methods as it is not practical for routine diagnosis (OIE technical disease cards, 2015 update). RHD can be reproduced using filtered and antibiotic-treated liver suspensions that are inoculated either by the intramuscular, intravenous or oronasal route. When the disease is clinically evident, the signs and *post-mortem* lesions are similar to those described after natural infection (OIE Terrestrial Manual 2016). When testing the *in-vivo* pathogenicity of RHDV or RHDVa the mortality rates (70–90%) are higher than with RHDV2 (20% on average, occurring later and over a longer period) (OIE Terrestrial Manual 2016).

The inability to grow lagoviruses *in vitro*, as well as most caliciviruses is thought to be associated with virus entry (e.g. receptor binding) (Guix et al., 2007)(Vashist et al., 2009). This issue is further addressed in **Study 1** of this chapter.

1.2.2. Indirect diagnostic methods

1.2.2.1. Serologic tests - antibody detection

Infection by RHDV can be diagnosed through detection of a specific antibody response. Due to the differential antigenic properties exhibited by RHDV and RHDV2 virus capsids, RHD serologic test should be based on the use of both antigens (OIE Terrestrial Manual 2016)(Bárcena et al., 2015). In the case of little or none anamnestic or epidemiological information, tests for both RHDV and RHDV2 should be performed and the results compared (OIE Terrestrial Manual 2016).

Three basic techniques are used for the serological diagnosis of RHDV, the haemagglutination inhibition (HI), indirect ELISA (I-ELISA) and competition ELISA (C-ELISA). HI is the simplest serologic method with regard to the availability of reagents and technical complexity. Both ELISAs are quicker and more suitable when a large number of samples are tested.

1.2.2.1.1. Haemagglutination inhibition (HI) test

The Haemagglutination inhibition (HI) test was the first reaction used for the detection of anti-RHD antibodies (Liu et al., 1984).

The antigen is prepared from fresh liver samples collected from infected rabbits. A liver homogenate is clarified by two consecutive low speed centrifugations and the supernatant is filtered through a 0.22 µm pore size mesh, titrated by HA, and divided into aliquots, which are stored at –70°C (OIE Terrestrial Manual 2016).

The serum samples under study must be pre-treated. First they are inactivated by heat and then treated with kaolin (25%) before centrifugation. After a second kaolin treatment the serum samples are adsorbed to the type "O" red blood cells to remove nonspecific activity (Capucci et al., 1991)(OIE Manual 1992). Sera are clarified by centrifugation. Pre-treating the sera improves the specificity of HI, but is time-consuming, limiting the number of samples that can be tested (21, 49, 61)(Capucci et al., 1991).

The serum titre is the end-point dilution showing inhibition of HA. The positive threshold of serum titres, usually in the range 1/20–1/80, is correlated to the titre of the negative control sera (OIE Terrestrial Manual 2016).

Again, the difficulty of obtaining and working with human Group “O” blood cells led to the substitution of the HI test by ELISA (OIE Terrestrial Manual 2016).

1.2.2.2. Enzyme-linked immunosorbent assay (ELISA)

1.2.2.2.1. Indirect ELISA (I-ELISA)

The I-ELISA was developed independently by Frescura et al. (1989), Rodák et al. (1990a) and Schirrmeier et al. (1990). It is used when a higher level of sensitivity is needed or to detect antibodies induced by cross-reacting non-pathogenic RCVs (Cooke et al., 2000).

The sera, serially diluted are incubated on a plate pre-coated with the antigen (purified RHDV), after which commercial enzyme conjugated immunoglobulin anti-rabbit IgG are added. The IgG bound to the antigen is detected using a reagent, preferably a MAb anti-rabbit IgG labelled HRPO. The titre of the serum corresponds to the highest dilution giving an absorbance value which is still considered positive. It is possible to test a single serum dilution to obtain a semi-quantitative estimation or to calculate its titre through a reference standard curve (Capucci et al., 1991).

The I-ELISA is a simpler technique and has higher sensitivity than C-ELISA, being suitable for measurement of highly cross-reactive antibodies and detection of antibodies with low avidity. With regards to specificity, The I-ELISA is more susceptible to false positives due to nonspecific antibodies (especially IgM, at low serum dilutions) that may bind to the solid phase (Capucci et al., 1991). Also, the direct adsorption the virus on the solid phase induces changes in the viral structure, resulting in the presentation of internal determinants and reducing the ability to discriminate between correlated viruses (Capucci et al., 1991).

1.2.2.2.2. Competition ELISA (C-ELISA)

C-ELISAs for RHDV were developed by Scicluna et al. (1990), and by Dr. Haas and Dr. Ronsholt (reviewed in Capucci et al., 1991). The protocols differ in the step at which competition takes place (Capucci et al., 1991). With regard to specificity, the C-ELISA has a markedly higher specificity than HI and I-ELISA (Capucci et al., 1991).

In the Scicluna et al. (1990) method, competition for the virus takes place during the first part of the reaction. After being directly diluted on the pre-coated plate, the sera are incubated with a prefixed concentration of RHDV antigen. The amount of specific antibodies present in the sera is then indirectly quantified by binding of HRPO conjugated rabbit IgG anti-RHDV. The serum titre is the dilution, reducing by 50% the absorbance value of the negative control. The serum is considered negative if the value of the selected dilution doesn't exceed 20% of the value of the negative control.

In the second method, the competition reaction starts after the absorption of the virus by the antiserum which coats the solid phase. A pre-set dilution of enzyme conjugated IgG anti-RHDV is added to the diluted sample sera. After the enzymatic reaction has taken place, the presence of specific antibodies in the sera is detected by the drop in absorbance with respect to the value of the negative control.

For C-ELISA, the antigen can be prepared as for HI but should be stored at -20ºC in glycerol (50% v/v) after being inactivated with 1.0% binary ethylenimine (BEI).

A specific polyclonal serum or anti-RHDV MAbs can be used. The specific polyclonal serum with high anti-RHDV or anti-RHDV2 titre can be obtained by various ways. Rabbits can be infected to obtain convalescent sera containing a high level of anti-RHDV IgG. The animals can be bleed between day 21 to 25 post infection or convalescent rabbits can be re-infected at 3–4 months post-infection and bled 10–15 days later to obtain RHDV hyperimmune sera. For RHDV2, sera of infected convalescent rabbits usually have a titre 20–40 times lower than that induced by RHDV, presumably due to the low mortality/high morbility induced. In alternative, the antigen (RHDV or RHDV2) can be purified from the livers of experimentally infected rabbits that died from an acute form of the disease (between 28 and 40 hours post-Infection), by the methods described by Capucci et al., (1991 and 1995) or Ohlinger et al., (1990). This antigen can be used to immunise sheep or goats according to classical protocols using oil adjuvants or rabbits, if the purified virus is inactivated before inoculation.

When anti-RHDV MAbs are used, the conjugated antibody (purified rabbit IgG and conjugation to HRPO) is titrated in a sandwich ELISA in the presence and absence of RHDV antigen (negative sample). The conjugated antibody is then used at the highest dilution showing maximum absorbance. The value of the HRPO conjugate should range from 1/1000 to 1/3000.

The negative control serum is obtained from rabbits fully susceptible to RHDV infection while the positive serum is either a convalescent serum diluted 1/100 in a negative serum or a serum collected from a vaccinated animal.

The serum is considered negative when the absorbance value of the first dilution (1/10) decreases by 15% of the reference value (dilution 1/10 of the negative control serum), while it is positive when the absorbance value decreases by 25% or more. When the absorbance value decreases by between 15% and 25% of the reference value, the sera is considered to be doubtful.

The serum titre corresponds to the dilution with an absorbance value equal to 50% (±10) of the average value of the three negative serum dilutions. This value is also suitable for C-ELISA with

RHDV2. The origin of the sample determines the range of titres found. In convalescent rabbits the positive sera range from 1/640 to 1/10,240, in vaccinated rabbits from 1/80 to 1/640 and "non-pathogenic" infection from 1/10 to 1/160. The origin of the sample determines if one or more dilutions should be tested.

An alternative C-ELISA method was described by Collins et al. (1995) using MAbs that were raised to a preparation of RHDV purified from the livers of experimentally infected rabbits.

1.2.2.2.3. Isotype enzyme-linked immunosorbent assays (isoELISAs)

The isoELISA assays enables the detection and titration of IgA, IgG and IgM isotypes, improving serological interpretation and classifying the immunological status of rabbits (Cooke et al., 2002). The isotype titres proved critical for the interpretation of field serology regarding the presence of cross-reactive antibodies (which can potentially confound serological data), the natural resilience of young rabbits, discrimination between temporary maternal antibodies and longer-lasting antibodies, and the presence of antibodies in previously infected rabbits (Cooke et al., 2000). Indeed, only IgG are detected in the case of passively aquired antibodies, with no IgA being detected in vaccinated animals, and first IgM and then IgA and IgG are detected in recently infected rabbits, (Cooke et al., 2000).

1.2.2.2.4. Other ELISAs

In Solid-phase ELISA (SP-ELISA), the purified antigen is directly adsorbed to the solid phase and because of virus deformation, internal epitopes are exposed. SP-ELISA detects a wider spectrum of RHDV antibodies and has high sensitivity and low specificity, and could be considered lagoviruses specific (OIE Terrestrial Manual, 2016).

The sandwich ELISA to detect IgM and IgG in liver or spleen samples is particularly useful in samples collected from animals that died from the chronic form of the disease, for which the detection of the virus may be difficult using HA or other ELISA methods. Samples are positive for RHD if a high level of RHDV-specific IgM and a low level, if any, of IgG are observed (OIE Terrestrial Manual, 2016).

1.2.2.2.5. Serological assays based on specific virus-like particles (VLPs)

Serological assays based on the use of virus specific VLPs enabling the discrimination between RHDV and the non-pathogenic rabbit caliciviruses RCV-A1 have also been developed (Liu et al., 2012)(Bárcena et al., 2015). Recently, the OIE Reference Laboratory also developed a serological assay based on specific anti-RHDV2 MAbs, rabbit immune serum and virus capsid antigen obtained from RHDV2-infected rabbit liver extracts (Camarda et al., 2014)(Bárcena et al., 2015)(OIE technical disease cards, 2015 update).

Study 1

Is RHDV2 cultivable and isolable in cell lines?

Rabbit haemorrhagic virus 2 (RHDV2) was not cultivable *in vitro* after 12 consecutive passages in RK13 and VERO cell lines, similarly to other lagoviruses

Carina L. Carvalho[1], Elsa L. Duarte[1,2], Margarida D. Duarte[3]

[1]Instituto de Ciências Agrárias e Ambientais Mediterrânicas (ICAAM), Universidade de Évora, Évora, Portugal; [2]Departamento de Medicina Veterinária, Universidade de Évora, Évora, Portugal; [3]Instituto Nacional de Investigação Agrária e Veterinária (INIAV), Lisboa, Portugal;

Manuscript in Preparation

1. Abstract

Rabbit haemorrhagic disease virus 2 (RHDV2) is a new pathogenic *Lagovirus* of the *Caliciviridae* family. Although a few caliciviruses such as the San Miguel sea lion virus (SMSV), the vesicular exanthema of swine virus (VESV) and the feline calicivirus (FCV) are adaptable to cell cultures, most cannot be propagated *in vitro*. Attempts to cultivate the genetically related, but distinct, classical RHDV date back to 1984 and its non-cultivability is largely known. However, to our knowledge, no studies have been published on the investigation of the potential ability of RHDV2 propagation on tissue culture cells.

In this study, we attempted to propagate RHDV2 in rabbit and monkey kidney cell lines, namely RK13 and VERO cells, by performing 12 consecutive passages, sub-cultured each 4 to 6 days. No cytopathogenic effect was observed during these cell passages, which suggests that RHDV2 did not infect and replicate in these cells. Corroborating these results, the supernatants from passages 3, 6, 9 and 12 tested negative for viral RNA by RT-qPCR. Viral RNA was detected in the supernatant of the first passage but the rapid decline and disappearance of the viral RNA indicated that it corresponded to the inoculum and that no viral replication occurred.

2. Keywords: Rabbit haemorrhagic disease, RHDV2, *in vitro* propagation, cell lines, RK13, VERO cells

3. Body of manuscript

Rabbit haemorrhagic disease virus 2 (RHDV2) is a new pathogenic virus of both wild and domestic rabbits belonging to genus *Lagovirus* of the *Caliciviridae* family.

The International Committee on Taxonomy of Viruses (ICTV) recognises five genera in the *Caliciviridae* family: *Lagovirus*, *Vesivirus*, *Norovirus*, *Sapovirus* and *Nebovirus* (Taube et al., 2010). The recently recognised genera *Recovirus*, which includes the bovine enteric virus Newbury agent-1 and the rhesus macaques Tulane virus, was also proposed to integrate the *Caliciviridae* family (Taube et al., 2010).

In general, caliciviruses exhibit a wide range of hosts, including humans, and tissue tropisms, causing a variety of diseases such as gastroenteritis (*Norovirus* and *Sapovirus*, the only genera infecting humans), haemorrhagic disease (*Lagovirus*) and vesicular lesions, respiratory infections and reproductive failure (*Vesivirus*) (Taube et al., 2010). The *Lagovirus* genus includes the classical RHDV as well as the recently emerged RHDV2 and the genetically related European brown hare

syndrome virus (EBHSV) (Capucci et al., 1996)(Le Gall-Reculé et al., 2011a)(Le Gall-Reculé et al., 2013), all responsible for high mortality rates in lagomorphs (Liu et al.,1984), and the non-pathogenic lagoviruses (Le Gall-Reculé et al., 2011b).

Caliciviruses are named after the characteristic cup-shaped depressions observed on the virions by negative stain electron microscopy (*Calyx*, Latin for chalice) (Clarke and Lambden, 1997). Although a few caliciviruses, such as the San Miguel sea lion virus (SMSV) and the vesicular exanthema of swine virus (VESV) (morphologically indistinguishable from each other) (Smith et al., 1973)(Sawyer, 1976), and the feline calicivirus (FCV) (Kreutz et al., 1994) are adapted to cell culture most of these viruses cannot be propagated in tissue culture cells (Clarke and Lambden, 1997)(Meyers et al., 2000)(Vashist et al., 2009). Cell cultures can be examined by optical microscope for evidences of viral proliferation, and have served as the gold standard for virus detection and the method to which all others have been compared (Leland and Ginocchio, 2007). However, attempts to adapt lagoviruses such as the EBHSV (Gavier-Widén and Mörner, 1991), RHDV (Parra and Prieto, 1990) and RCV (Capucci et al., 1996) to grow in various cell lines have repeatedly failed. The inability to grow *Lagovirus* and most caliciviruses *in vitro* is suggested to be associated with virus entry and receptor binding (Guix et al., 2007)(Vashist et al., 2009). Viruses can bind to many different molecules (proteins, lipids, and carbohydrates), some of which are attachment factors that concentrate virus on the cell surface while others are receptors or co-receptors that facilitate virus entry into cells (Taube et al., 2010). RHDV was the first calicivirus to show binding to histo-blood group antigens (HBGAs) of the upper respiratory and/or digestive tract epithelial cells (Ruvoën-Clouet et al., 2000), followed by noroviruses (Hutson et al., 2002)(Marionneau et al., 2002). HBGAs are attachment factors (ligands) that facilitate RHDV infection rather than the main cellular receptor (Nyström et al., 2011). They are polymorphic carbohydrate structures synthesised by a stepwise addition of monosaccharides to different precursor structures, via specific glycosyltransferases (Leuthold et al., 2015). HBGAs represent terminally exposed portions of larger glycans linked to proteins or glycolipids, expressed mainly on epithelial surfaces (Nyström et al., 2012) but also on red blood cells or in secreted fluids, such as saliva and mucins of the intestinal tract (Nystrom et al., 2011). They can be subdivided into ABH and Lewis antigens, of which at least four were found to interact with RHDV (A type 2, B and H type 2 and Lewis Y)(Leuthold et al., 2015). Classical G1 to G6 RHDVs were found to bind to HBGAs in a strain-dependent manner and with variable magnitudes (Nyström et al., 2011). The liver is considered the major organ of RHDV replication. However, rabbit hepatocytes are completely devoided of HBGAs (Gorvel et al., 1985)(Nyström et al., 2011) which

renders unlikely that RHDV uses HBGAs as receptors on hepatocytes, suggesting that additional mechanisms are implied in the RHDV infection (Nyström et al., 2011). RHDV infection has been shown to be rabbit specific despite cross-species recognition of HBGAs in human cells (Nyström et al., 2012), which indicates that other molecular elements exclusive to rabbits restrict its host range (Leuthold et al., 2015).

There is still limited information on the RHDV2 interactions with HBGAs. In a recent study on the RHDV binding to HBGAs, G6 group (RHDVa) and RHDV2 were found to have variable interactions with human blood types as the P domains of both viruses appeared markedly different (Leuthold et al., 2015). This data raises the hypothesis that RHDV2 may have different HBGA binding mechanisms (Leuthold et al., 2015).

Regarding RHDV2, HA assays using human O, A, B, and AB erythrocytes showed that this new virus, like G1 and G6, has variable interactions with the different blood types (Dalton et al., 2012) (Leuthold et al., 2015). While some authors refer that RHDV2 agglutinates human type "O" erythrocytes efficiently (Le Gall-Reculé et al., 2013), others state that RHDV2 displays a pattern similar to G4 and G6 groups with no agglutination of blood groups O or A, but agglutinating blood groups B and AB (Dalton et al., 2012).

The lack of a culture system for caliciviruses replication poses major obstacles for applied and basic research development, limiting the classification of these viruses, since several important features used to distinguish between families, such as physicochemical properties, protein synthesis in infected cells, antigenic relationships, and cell tropism, cannot be easily analysed in the absence of effective cell culture systems (Green et al., 2000). Studies on caliciviruses have therefore relied on the experimental infection of rabbits and on *in vitro* methods, including cDNA synthesis and cloning and sequencing analysis, using RNA extracted from viral particles or liver tissue of infected rabbits (Meyers et al., 1991)(Maniloff, 1995)(Green et al., 2000)(Meyers et al., 2000).

RHDV2 genome research has been carried out using RNA extracted from virus particles recovered from liver tissue of infected rabbits. RHDV2 is genetically related but distinct from the non-cultivable RHDV (Le Gall-Reculé et al., 2011a)(Le Gall-Reculé et al., 2013). The possibility that those genetic differences could impact on the cultivable nature of RHDV2 was addressed in this study, by attempting to propagate RHDV2 during 12 passages in VERO cell line, derived from the kidney epithelium of the African green monkey (*Cercopithecus aethiops*) (Rhim et al., 1969)(Macfarlane and Sommerville, 1969) and in RK-13, a rabbit kidney-13 cell line. VERO cells were chosen for their ability to support the growth of a wide range of viruses to high titres, showing extensive

cytopathogenic effects (CPE) (changes that range from swelling, shrinking, and rounding of cells to clustering, syncytium formation, and, in some cases, complete destruction of the monolayer (Leland et al., 2007)) and plaque formation which provide suitable source of virus and antigen for other studies (Rhim et al., 1969)(Macfarlane and Sommerville, 1969). The RK-13 culture is a stable line of rabbit kidney cells that has shown the ability to assemble and produce complete viral particles when transfected by an infectious RHDV clone (Liu et al., 2006).

Liver and spleen samples were obtained from four wild rabbit specimens found dead in the Alto Alentejo region that tested RHDV2-positive by RT-qPCR (Duarte et al., 2015) with low Cq values (6.88 to 10.30). Homogenates of liver samples (30% w/v) were prepared in phosphate-buffered saline solution (PBS). The homogenate was vortexed and large debris removed after centrifugation for 5 min at 3000 g.

VERO and RK13 cells were prepared from a frozen master cell bank. After a quick thaw at 35°C, 1 mL of cell suspension, containing approximately 106 cells, was seeded in 25 cm^2 flasks with 10 mL of growth medium (MEM, 10% heat-inactivated (56°C, 30 min) foetal bovine serum (FBS), 2 mML-glutamine and 100 U of streptomycin and neomycin) and incubated at 37°C with 5% CO_2. Upon confluence, cells were sub-cultured into 25 cm^2 bottles (Nunc).

For viral passages, the medium of sub-confluent cultures was removed and cells were inoculated with 200 µl of the clarified viral suspension prepared from the liver and the spleen of each animal, or or with 200 µl of supernatant of the previous passage. After addition of MEM supplemented with 10% FBS, glutamine and antibiotics at the concentrations described above, the cultures were incubated at 37°C in a 5% of CO_2 atmosphere. Control cultures were mock inoculated with the same volume of maintenance medium and further treated by the same procedures as virus-inoculated cultures. Inoculated and mock cell cultures were checked daily under optical microscopy for the presence of cytopathic effects (CPE), to monitor virus growth/replication. In the absence of CPE a second criteria was considered before rejecting a "negative" culture (Lednicky and Wyatt, 2012) namely by investigating the presence of infectious virus in the supernatants of passages number 3, 6 and 9 and 12 by molecular methods. For that purpose, RNA (10 µl) was extracted from a 200 µl sample of the clarified supernatant in a BioSprint 96 nucleic acid extractor (Qiagen, Hilden, Germany) according to the manufacturer's instructions, and assessed for RHDV2 by RT-qPCR (Duarte et al., 2015) using the One Step RT-PCR kit (Qiagen, Hilden, Germany). Undetectable Cq or Cq values >40 were considered negative.

After 12 consecutive passages, it was observed that RHDV2 neither grew in VERO nor in RK13 cell lines, as no CPE was observed. The supernatant of passages 3, 6, 9 and 12 tested RHDV2-negative by RT-qPCR.

The microscopic examination of degenerative morphologic changes in monolayer cells culture has long been the standard approach for detecting viral proliferation, and proved to be a suitable method to isolate a wide variety of viruses. Despite since the early 2000s, detection of viruses in clinical samples by molecular methods has become widely available, virus isolation continues to be a sensitive method for the detection of infectious virus and remains a useful approach in the diagnosis of viral diseases, being less costly and generally better suited for detecting a comprehensive range of viruses (Leland and Ginocchio, 2007). It also allows the verification of the Koch's postulates.

Recently, numerous innovations in cell culture formats have been developed, like cell monolayers in rapid culture cell tubes and in microwell plates, and new technologies, including cell growth in three dimensions, various new or engineered cell lines and primary cells for the propagation of viruses considered very difficult to study *in vitro*, blind Immunofluorescence (IF) or colour change reactions that enable viruses' identification pre-CPE detection (reviewed by Leland and Ginocchio, 2007)(Lednicky and Wyatt, 2012). Despite these advances, the lack of RHDV2 adaptation to cell lines is in accordance to the results obtained for other lagoviruses, as referred previously, namely for classical RHDV (Parra and Prieto, 1990), RCV (Capucci et al., 1996) or the EBHSV (Gavier-Widén et al., 1991).

In the future, other cell lines could be screened for attachment factors or receptors that may enhance viral replication towards the successfully propagation RHDV2. For instance, sialic acids (HBGAs associated) were identified as attachment factors for GII norovirus (Nystrom et al., 2011) (Taube at al., 2010). Considering that RHDV2 and genogroup II norovirus interact in a similar fashion with HBGAs, it may be possible that a GII norovirus susceptible cell culture could also work with RHDV2 (Leuthold et al., 2015). Also, transgenic technology offers the possibility of using genetically modified ("engineered") cell lines to improve virus growth in cell culture, facilitating virus-infected cells detection (Lednicky and Wyatt, 2012). When a virus receptor is present on the cell surface in a suboptimal number, genetic engineering of susceptible cell lines to over-express virus receptors is likely to improve virus attachment and entrance into the cell (Lednicky and Wyatt, 2012). For instance, VERO E6 cells have been engineered to over-express canine signalling

lymphocyte activation molecule (cSLAM), thought to be the major virus receptor of Canine Distemper Virus (CDV) (Lednicky and Wyatt, 2012).

Until the development of biological systems susceptible to increase the knowledge of the biological aspects of these viruses, the replication of lagoviruses is still greatly dependent on recombinant DNA technology, crucial for the production and characterization of viral proteins, and also the basis of molecular studies on the mechanisms of viral replication (Green et a., 2000)(Morales et al., 2004).

In the past, production of viral proteins in heterologous systems enabled the study of the three-dimensional structure of caliciviruses by cryo-electron microscopy and was proven useful for the development of viral vaccines and diagnostic strategies (Sibilia et al., 1995)(Nagesha et al., 1995)(Laurent et al., 1994). For instance, the RHDV capsid protein was successfully expressed in insect cells where it spontaneously assembled to form virus-like particles (VLPs), physically and immunologically indistinguishable from the intact wild-type virions (Sibilia et al., 1995)(Nagesha et al., 1995)(Laurent et al., 1994). This was particularly useful in the development of recombinant RHDV vaccines (Laurent et al., 1994)(Sibilia et al., 1995)(Plana-Duran et al., 1996).

Reverse genetics using the infectious clone technology was successfully applied to RHDV a decade ago (Liu, 2006). After the construction of an infectious full-length cDNA clone of RHDV and subsequent synthesis of infectious RNA in the transfected cells, recombinant viruses were generated. RNA transcripts showed to be infectious when inoculated to rabbits which died in the following 72 hours. Furthermore, the rescued virus presented 99.9% genetic homology to the parental virus and quantification by real time PCR showed a positive correlation between time of infection and virus titre (Liu et al., 2006). This method offered a more accurate approach to elucidate the mechanisms involved in viral pathogenesis *in vivo* (Liu et al., 2006). Latter, a similar investigation was carried out in vitro using RK13 cells (Liu et al., 2008). When RK13 cells were transfected with a full-length RHDV cDNA clone, CPE was evident at 12 h post-transfection and more prominent at 48 h post-transfection (Liu et al., 2008).

Reverse genetics proved to be a useful tool for studying viral RNA replication, pathogenesis and in vivo function of individual viral proteins, as well as for developing new vaccines against RHDV (Liu et al., 2006)(Liu et al., 2008).

Genome sequence analysis represents a major tool for the study of lagoviruses and other non-cultivable caliciviruses as well as for the clarification of the relationships among *Caliciviridae* family

members or virus groups containing non-cultivatable members, as recognized by the ICTV (Green et al., 2000).

Regarding RHDV2, further studies are needed to better understand the biological aspects of this relevant emergent virus. Recently, a recombinant baculovirus expressing the RHDV2 VP60 protein was also generated by Bárcena et al. (2015). VLPs may be useful for the development of recombinant subunit vaccines for RHDV2, considering that the provisionally authorised vaccines for RHDV2 are prepared from liver extracts of experimentally infected rabbits (Bárcena et al., 2015). Also, VLPs enabled the characterization of the monoclonal antibodies (MAbs) 2E7, 1G5 and 1C9, regarding their differential reactivity with three lagoviruses capsid proteins (RHDV, RHDV2 and EBHSV), providing valuable tools for monitoring virus circulation and for the development of control measures, and fundamental research in different aspects of the RHDV2 biology (Bárcena et al.. 2015).

4. Acknowledgments

The authors thank to Maria João Teixeira, Ricardino Ferreira, Fátima Cordeiro and Arminda da Conceição for their technical assistance. This study was partially funded by a grant from the Fundação para a Ciência e Tecnologia (FCT) SFRH/79225/2011.

5. References

Bárcena, J., Guerra, B., Angulo, I., González, J., Valcárcel, F., Mata, C.P., Castón, J.R., Blanco, E., Alejo, A., 2015. Comparative analysis of rabbit hemorrhagic disease virus (RHDV) and new RHDV2 virus antigenicity, using specific virus-like particles. Vet. Res. 46, 106. doi:10.1186/s13567-015-0245-5

Capucci, L., Fusi, P., Lavazza, A., Pacciarini, M.L., Rossi, C., 1996. Detection and preliminary characterization of a new rabbit calicivirus related to rabbit hemorrhagic disease virus but nonpathogenic. J. Virol. 70, 8614–23.

Clarke, I.N., Lambden, P.R., 1997. The molecular biology of caliciviruses. J. Gen. Virol. 78 (Pt 2), 291–301. doi:10.1099/0022-1317-78-2-291

Dalton, K.P., Nicieza, I., Balseiro, A., Muguerza, M.A., Rosell, J.M., Casais, R., Álvarez, Á.L., Parra, F., 2012. Variant rabbit hemorrhagic disease virus in young rabbits, Spain. Emerg. Infect. Dis. 18, 2009–12. doi:10.3201/eid1812.120341

Duarte, M.D., Carvalho, C.L., Barros, S.C., Henriques, A.M., Ramos, F., Fagulha, T., Luís, T., Duarte, E.L., Fevereiro, M., 2015. A real time Taqman RT-PCR for the detection of rabbit hemorrhagic disease virus 2 (RHDV2). J. Virol. Methods 219, 90–5. doi:10.1016/j.jviromet.2015.03.017

Gavier-Widén, D., Mörner, T., 1991. Epidemiology and diagnosis of the European brown hare syndrome in Scandinavian countries: a review. Rev. Sci. Tech. 10, 453–8.

Gorvel, J.-P., Rigal, A., Sarles, J., Maroux, S., 1985. Aminopeptidase N- and human blood group A-antigenicity along the digestive tract and associated glands in the rabbit. Cell Tissue Res. 239, 241–248. doi:10.1007/BF00214925

Green, K.Y., Ando, T., Balayan, M.S., Berke, T., Clarke, I.N., Estes, M.K., Matson, D.O., Nakata, S., Neill, J.D., Studdert, M.J., Thiel, H.J., 2000. Taxonomy of the caliciviruses. J. Infect. Dis. 181 Suppl , S322–30. doi:10.1086/315591

Guix, S., Asanaka, M., Katayama, K., Crawford, S.E., Neill, F.H., Atmar, R.L., Estes, M.K., 2007. Norwalk Virus RNA Is Infectious in Mammalian Cells. J. Virol. 81, 12238–12248. doi:10.1128/JVI.01489-07

Hutson, A.M., Atmar, R.L., Graham, D.Y., Estes, M.K., 2002. Norwalk Virus Infection and Disease Is Associated with ABO Histo–Blood Group Type. J. Infect. Dis. 185, 1335–1337. doi:10.1086/339883

Kreutz, L.C., Seal, B.S., Mengeling, W.L., 1994. Early interaction of feline calicivirus with cells in culture. Arch. Virol. 136, 19–34. doi:10.1007/BF01538814

Laurent, S., Vautherot, J.F., Madelaine, M.F., Le Gall, G., Rasschaert, D., 1994. Recombinant rabbit hemorrhagic disease virus capsid protein expressed in baculovirus self-assembles into viruslike particles and induces protection. J. Virol. 68, 6794–6798.

Le Gall-Reculé, G., Lavazza, A., Marchandeau, S., Bertagnoli, S., Zwingelstein, F., Cavadini, P., Martinelli, N., Lombardi, G., Guérin, J.-L., Lemaitre, E., Decors, A., Boucher, S., Le Normand, B., Capucci, L., 2013. Emergence of a new lagovirus related to Rabbit Haemorrhagic Disease Virus. Vet. Res. 44, 81. doi:10.1186/1297-9716-44-81

Le Gall-Reculé, G., Zwingelstein, F., Boucher, S., Le Normand, B., Plassiart, G., Portejoie, Y., Decors, A., Bertagnoli, S., Guérin, J.-L., Marchandeau, S., 2011a. Detection of a new variant of rabbit haemorrhagic disease virus in France. Vet. Rec. 168, 137–8. doi:10.1136/vr.d697

Le Gall-Reculé, G., Zwingelstein, F., Fages, M.-P., Bertagnoli, S., Gelfi, J., Aubineau, J., Roobrouck, A., Botti, G., Lavazza, A., Marchandeau, S., 2011b. Characterisation of a non-pathogenic and non-

protective infectious rabbit lagovirus related to RHDV. Virology 410, 395–402. doi:10.1016/j.virol.2010.12.001

Leland, D.S., Ginocchio, C.C., 2007. Role of cell culture for virus detection in the age of technology. Clin. Microbiol. Rev. 20, 49–78. doi:10.1128/CMR.00002-06

Lednicky, J., Wyatt, D., 2012. The Art of animal cell culture for virus isolation. Biomed. tissue Cult. 151–178. doi:10.5772/51215

Leuthold, M.M., Dalton, K.P., Hansman, G.S., 2015. Structural analysis of a rabbit hemorrhagic disease virus binding to histo-blood group antigens. J. Virol. 89, 2378–87. doi:10.1128/JVI.02832-14

Liu, G., Ni, Z., Yun, T., Yu, B., Chen, L., Zhao, W., Hua, J., Chen, J., 2008. A DNA-launched reverse genetics system for rabbit hemorrhagic disease virus reveals that the VP2 protein is not essential for virus infectivity. J. Gen. Virol. 89, 3080–3085. doi:10.1099/vir.0.2008/003525-0

Liu, G., Zhang, Y., Ni, Z., Yun, T., Sheng, Z., Liang, H., Hua, J., Li, S., Du, Q., Chen, J., 2006. Recovery of infectious rabbit hemorrhagic disease virus from rabbits after direct inoculation with in vitro-transcribed RNA. J. Virol. 80, 6597–602. doi:10.1128/JVI.02078-05

Macfarlane, D.E., Sommerville, R.G., 1969. VERO cells (cercopithecus aethiops kidney)? growth characteristics and viral susceptibility for use in diagnostic virology. Arch. fr die gesamte Virusforsch. 27, 379–385. doi:10.1007/BF01249659

Maniloff, J., 1995. Identification and classification of viruses that have not been propagated. Arch. Virol. 140, 1515–1520. doi:10.1007/BF01322679

Marionneau, S., Ruvoën, N., Le Moullac-Vaidye, B., Clement, M., Cailleau-Thomas, A., Ruiz-Palacois, G., Huang, P., Jiang, X., Le Pendu, J., 2002. Norwalk virus binds to histo-blood group antigens present on gastroduodenal epithelial cells of secretor individuals. Gastroenterology 122, 1967–77

Meyers, G., Wirblich, C., Thiel, H.-J., 1991. Genomic and subgenomic RNAs of rabbit hemorrhagic disease virus are both protein-linked and packaged into particles. Virology 184, 677–686. doi:10.1016/0042-6822(91)90437-G

Meyers, G., Wirblich, C., Thiel, H.J., Thumfart, J.O., 2000. Rabbit hemorrhagic disease virus: genome organization and polyprotein processing of a calicivirus studied after transient expression of cDNA constructs. Virology 276, 349–63. doi:10.1006/viro.2000.0545

Morales, M., Bárcena, J., Ramírez, M.A., Boga, J.A., Parra, F., Torres, J.M., 2004. Synthesis in vitro of rabbit hemorrhagic disease virus subgenomic RNA by internal initiation on (-)sense genomic RNA: mapping of a subgenomic promoter. J. Biol. Chem. 279, 17013–8. doi:10.1074/jbc.M313674200

Nagesha, H.S., Wang, L.F., Hyatt, A.D., Morrissy, C.J., Lenghaus, C., Westbury, H.A., 1995. Self-assembly, antigenicity, and immunogenicity of the rabbit haemorrhagic disease virus (Czechoslovakian strain V-351) capsid protein expressed in baculovirus. Arch. Virol. 140, 1095–1108. doi:10.1007/BF01315418

Nyström, K., Le Gall-Reculé, G., Grassi, P., Abrantes, J., Ruvoën-Clouet, N., Le Moullac-Vaidye, B., Lopes, A.M., Esteves, P.J., Strive, T., Marchandeau, S., Dell, A., Haslam, S.M., Le Pendu, J., 2011. Histo-blood group antigens act as attachment factors of rabbit hemorrhagic disease virus infection in a virus strain-dependent manner. PLoS Pathog. 7, e1002188. doi:10.1371/journal.ppat.1002188

Nyström, K., Le Moullac-Vaidye, B., Ruvoën-Clouet, N., Le Pendu, J., 2012. Shared Human/Rabbit Ligands for Rabbit Hemorrhagic Disease Virus. Emerg. Infect. Dis. 18, 518–519. doi:10.3201/eid1803.111402

Parra, F., Prieto, M., 1990. Purification and characterization of a calicivirus as the causative agent of a lethal hemorrhagic disease in rabbits. J. Virol. 64, 4013–4015

Plana-Duran, J., Bastons, M., Rodriguez, M.J., Climent, I., Cortes, E., Vela, C., Casal, I., 1996. Oral immunization of rabbits with VP60 particles confers protection against rabbit hemorrhagic disease. Arch Virol 141, 1423–36. doi:10.1007/BF01718245

Rhim, J.S., Schell, K., Creasy, B., Case, W., 1969. Biological Characteristics and Viral Susceptibility of an African Green Monkey Kidney Cell Line (Vero). Exp. Biol. Med. 132, 670–678. doi:10.3181/00379727-132-34285

Ruvoën-Clouet, N., Ganière, J.P., André-Fontaine, G., Blanchard, D., Le Pendu, J., 2000. Binding of rabbit hemorrhagic disease virus to antigens of the ABH histo-blood group family. J. Virol. 74, 11950–4.

Sawyer, J.C., 1976. Vesicular exanthema of swine and San Miguel sea lion virus. J. Am. Vet. Med. Assoc. 169, 707–9

Sibilia, M., Boniotti, M.B., Angoscini, P., Capucci, L., Rossi, C., 1995. Two independent pathways of expression lead to self-assembly of the rabbit hemorrhagic disease virus capsid protein. J. Virol. 69, 5812–5

Smith, A.W., Akers, T.G., Madin, S.H., Vedros, N.A., 1973. San Miguel Sea Lion Virus Isolation, Preliminary Characterization and Relationship to Vesicular Exanthema of Swine Virus. Nature 244, 108–110. doi:10.1038/244108a0

Taube, S., Jiang, M., Wobus, C.E., 2010. Glycosphingolipids as Receptors for Non-Enveloped Viruses. Viruses 2, 1011–1049. doi:10.3390/v2041011

Vashist, S., Bailey, D., Putics, A., Goodfellow, I., 2009. Model systems for the study of human norovirus biology. Future Virol. 4, 353–367. doi:10.2217/fvl.09.18

Study 2

Can a molecular method for the detection of RHDV2 be developed to assist diagnosis?

A real time Taqman RT-PCR for the detection of rabbit hemorrhagic disease virus variant 2 (RHDV2)

Margarida D. Duarte[1], Carina L. Carvalho[2], Sílvia S. Barros[1] , Ana M. Henriques[1], Fernanda Ramos[1], Teresa Fagulha[1], Tiago Luís[1], Elsa L. Duarte[2,3], Miguel Fevereiro[1]

[1]Instituto Nacional de Investigação Agrária e Veterinária (INIAV), Lisboa, Portugal; [2]Instituto de Ciências Agrárias e Ambientais Mediterrânicas (ICAAM), Universidade de Évora, Évora, Portugal; [3]Departamento de Medicina Veterinária, Universidade de Évora, Évora, Portugal;

Journal of Virologic Methods 219:90-5.

Journal of Virological Methods 219 (2015) 90–95

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

A real time Taqman RT-PCR for the detection of rabbit hemorrhagic disease virus 2 (RHDV2)

Margarida Dias Duarte[a,*], Carina L. Carvalho[b], Silvia C. Barros[a], Ana M. Henriques[a], Fernanda Ramos[a], Teresa Fagulha[a], Tiago Luís[a], Elsa L. Duarte[b], Miguel Fevereiro[a]

[a] Instituto Nacional de Investigação Agrária e Veterinária (INIAV), Rua General Morais Sarmento, 1500-310 Lisbon, Portugal
[b] Universidade de Évora, ICAAM/Escola de Ciências e Tecnologia, Apartado 92, 7002-554 Évora, Portugal

Article history:
Received 12 February 2015
Received in revised form 6 March 2015
Accepted 18 March 2015
Available online 28 March 2015

Keywords:
Rabbit hemorrhagic disease virus
RHDV2
RHDVb
Taqman RT-PCR
Real time RT-qPCR

ABSTRACT

A specific real time RT-PCR for the detection of RHDV2 was developed and validated using RHDV and RHDV2 RNA preparations from positive field samples. The system was designed to amplify a 127 nucleotide-long RNA region located within the *vp60* gene, based on the alignment of six sequences originated in Portugal, obtained in our laboratory, and 11 sequences from France and Italy.

The primers and probe target sequences are highly conserved in the vast majority of the RHDV2 sequences presently known. In the sequences showing variability, only one mismatch is found per strain, usually outlying the 3′ end of the primer or probe hybridization sequences.

The specificity of the method was demonstrated *in vitro* with a panel of common rabbit pathogens. Standardization was performed with RNA transcripts obtained from a recombinant plasmid harboring the target sequence. The method was able to detected nine RNA molecules with an efficiency of 99.4% and a R^2 value of 1. Repeatability and reproducibility of the method were very high, with coefficients of variation lower than 2.40%. The assay was proven a valuable tool to diagnose most of RDVH2 circulating strains, and may be also useful to monitor viral loads, and consequently, disease progression and vaccination efficacy.

© 2015 Elsevier B.V. All rights reserved.

1. Introduction

Rabbit haemorrhagic disease virus 2 (RHDV2) emerged in France in 2010 (Le Gall-Recule et al., 2011) and by the end of 2014 had already spread to Italy (Le Gall-Recule et al., 2013), Spain (Dalton et al., 2012), Germany (information on the FLI, 10|21|2013), Portugal mainland (Abrantes et al., 2013), England and Wales (Westcott et al., 2014) and Scotland (Baily et al., 2014). In 2015, RHDV2 was reported in several islands of the Azores (Duarte et al., 2015). RHDV2 shares with the highly pathogenic rabbit hemorrhagic disease virus (RHDV) the same genome structure, organized in two potential open reading frames (ORFs), and about 85% of the *vp60* nucleotide sequences (Dalton et al., 2015; Le Gall-Recule et al., 2013). It belongs to genus *Lagovirus* of the family *Caliciviridae*, along with the European brown hare syndrome virus (EBHSV), and affects European rabbits (*Oryctolagus cuniculus*)(Abrantes et al., 2013; Le Gall-Recule et al., 2013) hares (*Lepus capiensis* and *Lepus corsicanus*) (Camarda et al., 2014; Puggioni et al., 2013). Besides the genetic and antigenic differences with classical RHDV, RHDV2 can affect younger rabbits and the mortality rates are usually lower (Dalton et al., 2014; Le Gall-Recule et al., 2013). Although the clinical characteristics may differ in the two infections (Le Gall-Recule et al., 2011) anatomo-pathological features can be similar in both cases, leading to difficult differentiation. The molecular epidemiology of RHDV2 has revealed that this virus, also referred as RHDVb, is rapidly replacing the previously circulating classical strains in France, Spain and Portugal (Dalton et al., 2014; Le Gall-Recule et al., 2013; Lopes et al., 2014). For those reasons, a specific and quick laboratorial diagnosis of RHDV2 is very often required by the veterinarians to assist the control of the infection in rabbit industries. Since rabbit lagoviruses are not cultivable *in vitro* (Capucci et al., 1998; Wirblich et al., 1994), laboratory confirmation relays greatly on genome amplification and sequencing methods. Different molecular assays for the detection of RHDV have been described since the late 90's, including conventional RT-PCR assays (Ros Bascunana et al., 1997; Tham et al., 1999; Yang et al., 2008), immunocapture-RT-PCR (Le Gall-Reculé, 2001), real time multiplex RT-PCR (Gall et al., 2007), and more recently, loop-mediated isothermal amplification (Yuan

* Corresponding author. Tel.: +351 217115290; fax: +351 217115387.
E-mail address: margarida.duarte@iniav.pt (M.D. Duarte).

http://dx.doi.org/10.1016/j.jviromet.2015.03.017
0166-0934/© 2015 Elsevier B.V. All rights reserved.

Table 1
Nucleotide sequences and position within the vp60 gene of the RHDV2 primers and probe.

Oligomer	Nucleotide sequence (5′-3′)	Size of amplicon	Position in vp60 gene
RHDV2-F	TGGAACTTGGCTTGAGTGTTGA	127	1571–1592
RHDV2-R	ACAAGCGTGCTTGTGGACGG		1678–1697
RHDV2 probe	FAM-TGTCAGAACTTGTTGACATCCGCCC-TAMRA	–	1664–1640

et al., 2013) and SYBR green-based real-time PCR (Niedzwiedzka-Rystwej et al., 2013). None of these two last methods were however designed to specifically detect RHDV2 strains.

Here we describe a Taqman-probe-based real time PCR (RT-qPCR) designed for the specific detection of RHDV2 strains that provides a clear diagnosis response in less than 3 h. The method is in use in our laboratory since early 2014.

2. Materials and Methods

2.1. RNA isolation

Liver and lung samples were homogenized in phosphate-buffered saline (PBS) to a final concentration of 30% (w/v). The homogenate was vortexed and centrifuged for 5 min at 3000 g. Total RNA was extracted from 200 μl of the supernatant using a BioSprint 96 nucleic acid extractor (Qiagen, Germany), according to the manufacturer's protocol and eluted in 100 μl of RNase-free water.

2.2. Designing of PCR primers and probe

Primers (RHDV2-forward and RHDV2-reverse) and probe (RHDV2-probe) were designed manually based on conserved regions evidenced on the alignment of *vp60* complete RHDV2 sequences from Portugal, France and Italy. The nucleotide sequences and positions in the *vp60* gene are illustrated in Table 1.

2.3. Real time RT-PCR optimization

The RT-qPCR was performed using a CFX-96 real time system (Bio-Rad) in a 96-well optical plate format. Amplification was carried out in 25 μl volume reactions using the OneStep RT-PCR Kit (Qiagen, Germany) according to the manufacturer's recommendations. Optimal assay performance was obtained using final concentrations of 1 μM and 0.2 μM of each primer and probe (NZYTech Ltd, Portugal), respectively. Thermal cycling conditions included one cycle at 50 °C for 45 min for reverse transcription, one cycle at 95 °C for 15 min for Taq polymerase activation and 50 cycles of cDNA amplification (95 °C for 15 s, 60 °C for 30 s and 72 °C for 30 s). Fluorescence was acquired during each extension step. Negative controls contained PCR-grade water.

2.4. Cloning and sequencing of the 127 bp-long RT-qPCR target sequence

A 127 bp fragment was amplified from a field strain (1017PT13, accession number KJ683896) using the designed primer pair (Table 1). The amplicon was cloned into the pCR2.1 TA vector using One Shot TOP10, chemically competent *Escherichia coli* (Invitrogen Corporation, San Diego, CA). Plasmid DNA was extracted from overnight Luria Broth supplemented with kanamycin *Escherichia coli* cultures grown at 37 °C, using the standard boiling DNA purification protocol (Holmes and Quigley, 1981). The presence and correct orientation of the insert was confirmed by *EcoR*I (New England Biolabs, UK) hydrolysis and sequencing analysis in a 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) using the BigDye™ Terminator v1.1 Cycle Sequencing Kit (Life technologies, Foster City, CA). Sequences were assembled with Seqscape Software v2.7 (Applied Biosystems, Foster City, CA, USA). Recombinant DNA was extracted from a 50 ml overnight LB culture using the plasmid midi purification kit (Qiagen, Germany). DNA was quantified with a NanoDrop 1000 (Thermo Scientific, USA).

2.5. In vitro *transcription and treatment of RNA transcripts with DNase I*

Linearization of p1017PT13-2 with *BamH*I (New England Biolabs, UK) was confirmed by agarose gel electrophoresis analysis. The band corresponding to the linearized DNA was excised, purified with the NZYTech column kit (NZYTech Ltd, Portugal), and quantified as described above.

In vitro transcription was performed using the MAXIscript kit (Ambion, UK) according to the manufacturer's instructions. Transcribed RNA was treated with DNase I recombinant, RNase-free (Roche, Germany), purified with the QIAamp Viral RNA Mini kit (Qiagen, Germany) and tested for the presence of DNA using RHDV2 primers and probe and the High Fidelity PCR Mix (Qiagen, Germany), according to the manufacturer's recommendations.

After confirmation of DNA absence, RNA transcripts were stored at −80 °C until use.

2.6. Standardization of the method

RNA concentration was determined using a NanoDrop 1000 (Thermo Scientific, USA). Tenfold dilutions series, ranging from 10^{-2} to 10^{-13}, of in vitro transcribed RNA were prepared in RNase-free water. Each dilution was tested by RT-qPCR in triplicate. The calibration curves were generated by the CFX Manager™ Software (Bio-Rad, USA). For each standard, the logarithm of the RNA copy number was plotted against the crossing point values (Cq values). The number of RNA molecule copies in each PCR reaction was calculated using the molecular mass of the RNA transcript and the amount of RNA (g) present in the amplification reactions, accordingly to the formula:

RNA copy number in the amplification reaction = amount of RNA (g) in the reaction/[Molecular mass of the transcribed RNA/6.022×10^{23}].

The molecular mass of one RNA molecule (245 nucleotides) was determined using the software conversor (http://www.changbioscience.com/genetics/mw.html).

The amplification efficiency was determined with the equation $E = [10\,(-1/k) - 1]$, were (k) is the slope of the linear regression.

2.7. Specificity evaluation

The specificity of the RT-qPCR assay for the detection of RHDV2 was evaluated with RNA preparations from RHDV2 and RHDV field strains, previously characterized at INIAV, feline calicivirus RNA and DNA from common rabbit pathogens (*Pasteurella multocida, Bordetella bronchiseptica*, myxoma virus and four species of genus *Eimeria*). The Eimeria samples were lysed using the Thermo Electron FastPrep FP120 Cell Disrupter (San Jose, CA, USA) and protease-treated prior to DNA extraction.

Table 2
Localization of single mismatches in the hybridization sequences of the primers and probe, regarding the RHDV2 vp60 nucleotide sequences presently available in GenBank.

Country of origin	No of sequences known	Year of sample collection	Year of sequence discloser	Mismatches			Source of data	Variable nucleotide (5′-3′)
				RHDV2-F	Probe	RHDV2-R		
France	5	2010	2013	0	0	0	FR819781, HE800530-532, HE819400	–
France	1	2010	2013	1	0	0	HE800529	TGGAACTTGGCTTGAGTGTCGA
Italy	5	2011	2013	0	0	0	JQ929052, KC345611-613, JX106023	–
Spain	1	2011	2014	0	0	0	JX133161	–
Italy	3	2012	2014	0	0	0	JX106022, KC907712, KC741409	–
Portugal[a]	11	2013	-	0	0	0	*This study*	–
Portugal[a]	1	2013	2015	0	0	0	KM115680	–
Portugal[a]	1	2013	2015	0	1	0	KM115711	TATCAGAACTTGTTGACATCCGCCC
Portugal[a]	3	2013	2015	0	0	1	KM115677, KM115678, KM115679	ACAAGCGTGCTTGTGGACGG
Portugal[a]	9	2014	–	0	0	0	*This study*	–
Portugal[a]	38	2014	2015	0	0	0	KM115667-675, KM115681-697, KM115699-710	–
Portugal[a]	2	2014	2015	1	0	0	KM115675-676	TGGAACTCGGCTTGAGTGTTGA
Portugal[a]	1	2014	2015	0	1	0	KM115698	TTTCAGAACTTGTTGACATCCGCCC
Portugal[a]	5	2014	2015	0	1	0	KM115712-716	TGTCAGGACTTGTTGACATCCGCCC
Portugal[a]	3	2015	–	0	0	0	*This study*	–

[a] Mainland.

2.8. Repeatability and Reproducibility

To assess intra-assay variability, each dilution was tested in triplicate in the same run. The mean Cq values, standard deviation (SD) and percent coefficient of variation (%CV) were calculated independently for each RNA dilution.

The inter-assay variability was evaluated in three independent runs, performed in different days and in different thermocyclers. The mean, standard deviation and coefficient of variation were calculated with all the Cq values obtained for each dilution in each run. The range (minimum and maximum values) for each parameter was determined.

3. Results

3.1. Specificity of the real time RT-PCR assay

In *silico* analysis against the GenBank database showed that the primers and probe target sequences are conserved in the great majority of strains that circulate since 2010 (Table 2). Single nucleotide variations were, however, detected in 12 strains disclosed recently (accessed 30 January, 2015) either in the forward and the reverse primer or in the probe hybridization sequence. Exception made for 3 strains where a single mismatch is found more close to the 3′ end of the primer reverse, most of these variations are located at 15 bases from the 3′ end of the primer or 19 or 23 bases from the 3′ end of the probe (Table 2).

In the *vp60* sequences from non-RHDV2 lagoviruses, several mismatches were found either in classical RHDV strains as in apathogenic or low pathogenic rabbit calicivirus (RCV) strains. The lowest level of discrepancy was observed in a reduced number of sequences from apathogenic RCV strains and comprehends a total of six mismatches, two of each located in the probe targeting sequence, abrogating amplification. In the vast majority of the RHDV and apathogenic RCV sequences, the number of mismatches was significantly higher (up to 7 mismatches in the probe and up to 7 and 6 mismatches in the forward and reverse primer, respectively).

The analytical specificity of RT-qPCR was evaluated *in vitro* with RHDV2 RNA-positive samples, previously diagnosed in INIAV laboratory by conventional PCR and sequencing. All RHDV2 samples were detected demonstrating the specificity of the assay. Consistently, neither fluorescence nor amplification was observed when the system was used to test nucleic acids from the common rabbit pathogens described above, confirming the absence of unspecific amplification due to cross-reactivity and/or fluorescence emission (Table 3). The negative controls, containing PCR-grade water instead of RNA template, never crossed the threshold line.

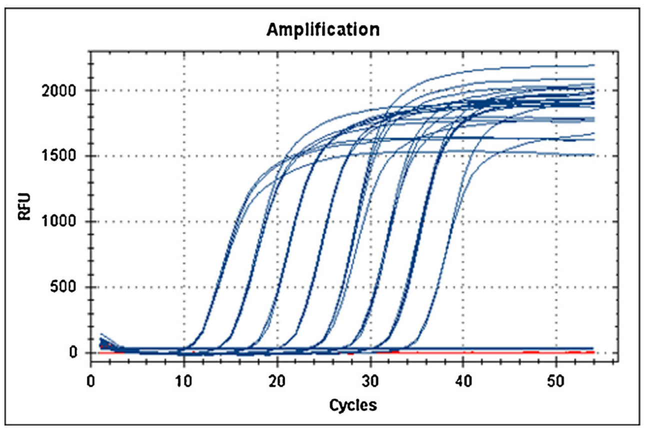
3.2. Standard curves

The method was standardized with serial dilutions of RNA transcribed *in vitro* from recombinant p1017P13-2. The absence of contaminant DNA after treating of the RNA synthetic transcripts with DNase I was confirmed by PCR with RHDV2 primers, since no amplification was obtained.

The robustness of the quantitative RT-qPCR was evidenced by the consistency of the data from independent regression analysis experiments. The slope values obtained ranged from 3.29 and 3.38, and the y-intercept values were lower than the cycle threshold [Cq] of 50, varying between 39.74 and 43.81. Efficiencies were never lower than 97.8%.

Table 3
Specificity evaluation of the RHDV2 RT-qPCR.

Pathogen	Samples tested	
	Strain/serotype	Result of RT-qPCR (Cq value)
RHDV2	1017PT13	14.03
	7285PT13	11.21
	16747PT13	14.15
RHDV	G1	No Cq
	G5	No Cq
	G6	No Cq
Myxoma virus	–	No Cq
Feline calicivirus	–	No Cq
Bordetella bronchiseptica (pure culture)		No Cq
Pasteurella multocida (pure culture)		No Cq
Eimeria stiedae		No Cq
Eimeria media		No Cq
Eimeria perforans		No Cq
Eimeria irresidua		No Cq



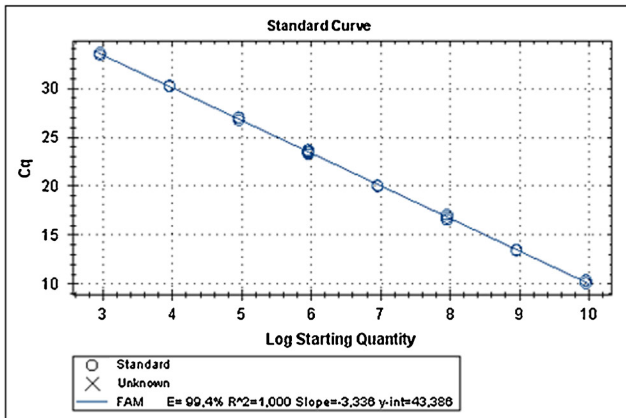


Fig. 1. Amplification and standard curves of RT-qPCR using RHDV2 RNA synthesized *in vitro* from a recombinant plasmid p1017PT-13. Standard curve was generated from the Cq values obtained against the log of known copy numbers (dilution series corresponding to 9.00E + 09 to 9.00E + 02 copies of RHDV2 RNA per reaction).

A typical standard curve amplification plot and linear regression analysis is shown in Fig. 1. Excellent linearity was observed over eight orders of magnitude, from dilution 10^{-2} (9.00e + 09 copies) to 10^{-9} (9.00e + 02 copies). The regression analysis for this interval yielded a R^2 (correlation coefficient) of 1 and a γ-intercept value of 43.54. The slope of 3.34 reveals a high RT-PCR efficiency (99.4%) closely approximating the amplification efficiency of 100% (3.32 slope).

3.3. Detection limit, repeatability and reproducibility of the real time RT- PCR assay

All replicates until dilution 10^{-11} tested positive, indicating that the method is able to detect nine copies of viral RNA (1.15e-18 g).

Intra-assay variability was calculated by assessing the homogeneity among replicates of dilutions 10^{-2} to 10^{-11}. The mean Cq values, SD values and % CV obtained disclosed very low variation (% CV ranging between 0.17 and 2.39) indicating the high repeatability of the method (Table 4).

Table 4
Intra- and inter-assay variability of the RT-qPCR.

Variation	Dilutions	Crossing point		
		Mean Cq	SD	% CV
Intra-assay[a]	10^{-2}	10.24	0.21	2.01
	10^{-3}	13.50	0.04	0.30
	10^{-4}	16.62	0.16	0.97
	10^{-5}	19.98	0.14	0.70
	10^{-6}	23.57	0.12	0.51
	10^{-7}	26.93	0.14	0.53
	10^{-8}	30.22	0.05	0.17
	10^{-9}	33.52	0.15	0.44
	10^{-10}	37.32	0.82	2.19
	10^{-11}	37.56	0.90	2.39
Inter-assay[b]	10^{-2}	10.04–10.96	0.01–0.21	0.06–2.01
	10^{-3}	13.36–13.86	0.04–0.17	0.30–1.29
	10^{-4}	16.62–16.80	0.16–0.23	0.97–1.36
	10^{-5}	19.98–20.35	0.13–0.18	0.65–0.90
	10^{-6}	21.51–24.14	0.04–0.23	0.15–0.99
	10^{-7}	26.93–27.13	0.07–0.14	0.24–0.53
	10^{-8}	30.20–30.22	0.05–0.16	0.17–0.54
	10^{-9}	33.53–34.19	0.15–0.16	0.44–0.46
	10^{-10}	37.32–38.25	0.10–0.82	0.25–2.19
	10^{-11}	37.55–37.56	0.90–1.03	2.39–2.73

SD – Standard deviation.
% CV – Percent coefficient variation.
[a] Assays were carried with triplicates.
[b] Values refer to three independent experiments.
The dilutions shown in the standard curve are highlighted.

Also, the elevated reproducibility of the assay was evidenced by the SD and the % CV obtained with the set of values from three independent assays, each using triplicates of the dilutions 10^{-2} to 10^{-11} (Table 4). The assay proved to be robust, showing percent coefficients of variation ranging between 0.06% and 2.39%, in different days or repetitions.

4. Discussion and conclusions

Since its first detection in 2010, RHDV2 has been spreading rapidly and replacing the classical RHDV genogroups circulating in wild rabbit populations in the Iberian Peninsula (Dalton et al., 2014; Delibes-Mateos et al., 2014; Lopes et al., 2014), France (Le Gall-Recule et al., 2013), Sardinia-Italy (Le Gall-Recule et al., 2013; Puggioni et al., 2013) and more recently, in Azores (Duarte et al., 2015).

The impact of RHDV2 infections in the rabbit industry has also increased in Portugal (Duarte et al., *unpublished data*) and Spain (Dalton et al., 2014; Dalton et al., 2012). In response to the epidemiologic dominance of RHDV2 strains over the former classical RHDV strains, and since the “old” vaccines only confer partial protection against RHDV2 infection (Dalton et al., 2014; Le Gall-Recule et al., 2013; Le Gall-Recule et al., 2011), several RHDV2 inactivated vaccines were developed in France and Spain and vaccination against this virus is becoming a common practice in the industry.

To assist the control of the disease, laboratorial confirmation was frequently requested to our institute, prompting us to develop a rapid and sensitive technique for detecting RHDV2 with high sensitivity and specificity. Molecular assays based on RT-PCR have been long used for the detection of RHDV classical genogroups, but to our knowledge, this report describes for the first time a Taqman-based RT-PCR for RDHV2 specific detection.

It has been suggested that RHDV2 emerged from a different species, yet unidentified (Le Gall-Recule et al., 2013) and is still adapting to its recent host, the European rabbit, and possibly also to the Cape hare, where disease is also induced (Puggioni et al., 2013). The immune pressure imposed by the new host/hosts to this naked virus, should impact mainly on the capsid protein-encoding gene leading to the accumulation of nucleotide variability. The maximum diversity at nucleotide and amino acid levels observed so far among RHDV2 vp60 complete sequences is 3.91% and 2.94% respectively and will eventually increase in the future.

The analytic specificity of the method was further demonstrated since sequencing of the VP60 carboxyl-terminal encoding domain from all the samples received at INIAV since 2013, confirmed that the RT-qPCR-positive strains belonged to the RHDV2 group.

The analysis of the vp60 sequences characterized in our laboratory or currently available in public databases ($n = 85$) showed that the regions targeted by the RT-qPCR primers and probe are 100% conserved in 85.9% of vp60 sequences from RHDV2 strains. Single mismatches were detected in the forward and reverse primers or in the probe hybridization sequences of 12 strains (Table 2). While single mismatches have been described to cause failure in the detection of respiratory syncytial virus (Whiley and Sloots, 2006) and West Nile virus (Papin et al., 2004), it has also been demonstrated that single SNP may not completely prevent amplification, although they may cause inefficient annealing and amplification and underestimation of the copy number (Lefever et al., 2013).

In four strains (HE800529, KM115677, KM115678, KM115679) the single mismatch can affect extension since it occurs at three bases from the 3′ end of the reverse primer. Taken into consideration the reduced number of strains with this variation, the Taqman probe-based assay described here constitutes the best compromise to detect RHDV2 strains. In fact, none of the other mismatches map within the last 4 or 5 bases of the 3′ end of the primer or probe, as the nucleotide substitutions are located 15 bases from the 3′ end of the forward primer or 19 and 23 bases from the 3′ end of the probe, limiting the impact on DNA polymerase extension (Lefever et al., 2013; Wu, Hong, and Liu, 2009), (Table 2). Moreover, a significant reduction in Tm and shift in Cq was observed when SNPs occur in both primers, or when more than one mismatch is present in a primer (Lefever et al., 2013), which is neither the case since no more than one mismatch was detected in a given RHDV2 strain regarding the primers and probe hybridization sequences.

Since RHDV2 is a RNA virus prone to evolution, continuous molecular surveillance is necessary to update the molecular tools for the detection of new descendant viral strains. This general requirement is applicable to all molecular methods designed to detect variants of any pathogen, since the accumulation of nucleotide mismatches may abrogate amplification.

In conclusion, the method developed is a fast, one step, sensitive RT-qPCR, useful for the detection of RHDV2 RNA, with the advantage over other real time PCR methods such as multiplex-PCR or SYBR-Green-based PCR, of conferring a highly specific and read-through signal generated by the Taqman probe. Furthermore, the highly sensitivity of the assay was verified by its ability to detect as few as nine molecules of RHDV2 RNA, allowing a rapid and conclusive laboratorial diagnosis in less than 3 h. When testing samples from animals in acute stages of infection, the Cq values obtained were systematically low (mean Cq values of 15.7 ± 1.2, mostly adults). Interestingly, the mean amount of virus found in rabbit kittens was 15,000 genome copies per mg liver (Matthaei et al., 2014). Taking into consideration that the amount of virus described in adult rabbits was found to be 1000-times higher when compared to kittens (Strive et al., 2010) the value estimated from the standard curve for our field samples (mean Cq value of 15.70) is in full agreement with this expectation (1.5×10^7 copies per mg of tissue). The average viral load, extrapolated for our field samples, is also concordant with the data obtained by Tunon et al. (Tunon et al., 2011) who reported 10^6 to 10^7 copies per mg of liver for adult rabbits infected with RHDV (strain AST/89).

The method described here constitutes therefore not only a valuable tool for rapid, sensitive and specific diagnosis, but also a mean to assess viral load measurements, which can be used to monitor disease progression and evaluate vaccination efficiency.

Acknowledgments

The authors thank Dr Teresa Albuquerque and Dr Ana Botelho, Bacteriology Laboratory of Instituto Nacional de Investigação Agrária e Veterinária (INIAV) and Dr Jacinto Gomes, Parasitology Laboratory, INIAV, for providing *Pasteurella, Bordetella* and *Coccidea* samples used in the cross reactivity investigation. The authors are also thankful to Maria João Teixeira and Fátima Cordeiro for their technical assistance. This study was partially funded by a grant from the Fundação para a Ciência e Tecnologia (FCT) SFRH/79225/2011.

References

Abrantes, J., Lopes, A.M., Dalton, K.P., Melo, P., Correia, J.J., Ramada, M., Alves, P.C., Parra, F., Esteves, P.J., 2013. New variant of rabbit hemorrhagic disease virus, Portugal, 2012-2013. Emerg Inf Dis 19, 1900–1902.

Baily, J.L., Dagleish, M.P., Graham, M., Maley, M., Rocchi, M.S., 2014. RHDV variant 2 presence detected in Scotland. Vet Rec 174, 411.

Camarda, A., Pugliese, N., Cavadini, P., Circella, E., Capucci, L., Caroli, A., Legretto, M., Mallia, E., Lavazza, A., 2014. Detection of the new emerging rabbit haemorrhagic disease type 2 virus (RHDV2) in Sicily from rabbit (Oryctolagus cuniculus) and Italian hare (Lepus corsicanus). Res Vet Sci 97, 642–645.

Capucci, L., Fallacara, F., Grazioli, S., Lavazza, A., Pacciarini, M.L., Brocchi, E., 1998. A further step in the evolution of rabbit hemorrhagic disease virus: the appearance of the first consistent antigenic variant. Virus Res 58, 115–126.

Dalton, K.P., Abrantes, J., Lopes, A.M., Nicieza, I., Alvarez, A.L., Esteves, P.J., Parra, F., 2015. Complete genome sequence of two rabbit hemorrhagic disease virus variant b isolates detected on the Iberian Peninsula. Arch Virol 160, 877–881.

Dalton, K.P., Nicieza, I., Abrantes, J., Esteves, P.J., Parra, F., 2014. Spread of new variant RHDV in domestic rabbits on the Iberian Peninsula. , 67–73.

Dalton, K.P., Nicieza, I., Balseiro, A., Muguerza, M.A., Rosell, J.M., Casais, R., Alvarez, A.L., Parra, F., 2012. Variant rabbit hemorrhagic disease virus in young rabbits, Spain. Emerg Infec Dis 18, 2009–2012.

Delibes-Mateos, M., Ferreira, C., Carro, F., Escudero, M.A., Gortazar, C., 2014. Ecosystem effects of variant rabbit hemorrhagic disease virus, Iberian Peninsula. Emerg Infec Dis 20, 2166–2168.

Duarte, M., Henriques, M., Barros, S.C., Fagulha, T., Ramos, F., Luis, T., Fevereiro, M., Benevides, S., Flor, L., Barros, S.V., Bernardo, S., 2015. Detection of RHDV variant 2 in the Azores. Vet Rec 176, 130.

Gall, A., Hoffmann, B., Teifke, J.P., Lange, B., Schirrmeier, H., 2007. Persistence of viral RNA in rabbits which overcome an experimental RHDV infection detected by a highly sensitive multiplex real-time RT-PCR. Vet Microbiol 120, 17–32.

Holmes, D.S., Quigley, M., 1981. A rapid boiling method for the preparation of bacterial plasmids. Anal Biochem 114, 193–197.

Le Gall-Recule, G., Lavazza, A., Marchandeau, S., Bertagnoli, S., Zwingelstein, F., Cavadini, P., Martinelli, N., Lombardi, G., Guerin, J.L., Lemaitre, E., Decors, A., Boucher, S., Le Normand, B., Capucci, L., 2013. Emergence of a new lagovirus related to Rabbit Haemorrhagic Disease Virus. Vet Res 44, 81.

Le Gall-Recule, G., Zwingelstein, F., Boucher, S., Le Normand, B., Plassiart, G., Portejoie, Y., Decors, A., Bertagnoli, S., Guerin, J.L., Marchandeau, S., 2011. Detection of a new variant of rabbit haemorrhagic disease virus in France. Vet Rec 168, 137–138.

Le Gall-Reculé, G., Zwingelstein, F., Portejoie, Y., Le Gall, G., 2001. Immunocapture-RT-PCR assay for detection and molecular epidemiology studies of Rabbit Haemorrhagic Disease and European Brown Hare Syndrome viruses. J Virol Methods 97, 49–57.

Lefever, S., Pattyn, F., Hellemans, J., Vandesompele, J., 2013. Single-nucleotide polymorphisms and other mismatches reduce performance of quantitative PCR assays. Clin Chem 59, 1470–1480.

Lopes, A.M., Correia, J., Abrantes, J., Melo, P., Ramada, M., Magalhaes, M.J., Alves, P.C., Esteves, P.J., 2014. Is the New Variant RHDV Replacing Genogroup 1 in Portuguese Wild Rabbit Populations? Viruses 7, 27–36.

Matthaei, M., Kerr, P.J., Read, A.J., Hick, P., Haboury, S., Wright, J.D., Strive, T., 2014. Comparative quantitative monitoring of rabbit haemorrhagic disease viruses in rabbit kittens. Virol J 11, 109.

Niedzwiedzka-Rystwej, P., Hukowska-Szematowicz, B., Dzialo, J., Tokarz-Deptula, B., Deptula, W., 2013. Real time PCR detection of rabbit haemorrhagic disease virus in rabbits infected with different European strains of RHDV. Pol J Vet Sci 16, 39–43.

Papin, J.F., Vahrson, W., Dittmer, D.P., 2004. SYBR green-based real-time quantitative PCR assay for detection of West Nile Virus circumvents false-negative results due to strain variability. J Clin Microbiol 42, 1511–1518.

Puggioni, G., Cavadini, P., Maestrale, C., Scivoli, R., Botti, G., Ligios, C., Le Gall-Recule, G., Lavazza, A., Capucci, L., 2013. The new French 2010 Rabbit Hemorrhagic Disease Virus causes an RHD-like disease in the Sardinian Cape hare (Lepus capensis mediterraneus). Vet Res 44, 96.

Ros Bascunana, C., Nowotny, N., Belak, S., 1997. Detection and differentiation of rabbit hemorrhagic disease and European brown hare syndrome viruses by amplification of VP60 genomic sequences from fresh and fixed tissue specimens. J Clin Microbiol 35, 2492–2495.

Strive, T., Wright, J., Kovaliski, J., Botti, G., Capucci, L., 2010. The non-pathogenic Australian lagovirus RCV-A1 causes a prolonged infection and elicits partial cross-protection to rabbit haemorrhagic disease virus. Virology 398, 125–134.

Tham, K.M., Barnes, S.M., Hunter, S.N., 1999. Polymerase chain reaction amplification and gene sequence analysis of a calicivirus from a feral rabbit. Virus genes 18, 235–242.

Tunon, M.J., San Miguel, B., Crespo, I., Riezu-Boj, J.I., Larrea, E., Alvarez, M., Gonzalez, I., Bustos, M., Gonzalez-Gallego, J., Prieto, J., 2011. Cardiotrophin-1 promotes a high survival rate in rabbits with lethal fulminant hepatitis of viral origin. J Virol 85, 13124–13132.

Westcott, D.G., Frossard, J.P., Everest, D., Dastjerdi, A., Duff, J.P., Steinbach, F., Choudhury, B., 2014. Incursion of RHDV2-like variant in Great Britain. Vet Rec 174, 333.

Whiley, D.M., Sloots, T.P., 2006. Sequence variation can affect the performance of minor groove binder TaqMan probes in viral diagnostic assays. J Clin Virol: the official publication of the Pan American Society for Clinical Virology 35, 81–83.

Wirblich, C., Meyers, G., Ohlinger, V.F., Capucci, L., Eskens, U., Haas, B., Thiel, H.J., 1994. European brown hare syndrome virus: relationship to rabbit hemorrhagic disease virus and other caliciviruses. J Virol 68, 5164–5173.

Wu, J.H., Hong, P.Y., Liu, W.T., 2009. Quantitative effects of position and type of single mismatch on single base primer extension. J Microbiol Meth 77, 267–275.

Yang, L., Wang, F., Hu, B., Xue, J., Hu, Y., Zhou, B., Wang, D., Xu, W., 2008. Development of an RT-PCR for rabbit hemorrhagic disease virus (RHDV) and the epidemiology of RHDV in three eastern provinces of China. J Virol Methods 151, 24–29.

Yuan, D., Guo, D., Liu, J., Si, C., Jiang, Q., Lin, H., Yang, T., Qu, L., 2013. Development of a reverse-transcription loop-mediated isothermal amplification method for detection of rabbit hemorrhagic disease virus. J Virol Methods 187, 274–277.

Study 3

What is the meaning of viral charges in the diagnosis of field samples?

Challenges in the rabbit haemorrhagic disease 2 (RHDV2) molecular diagnosis of vaccinated rabbits

Carina L. Carvalho[1], Elsa L. Duarte[1,2], Madalena Monteiro[3], Ana Botelho[3], Teresa Albuquerque[3], Miguel Fevereiro[3], Ana M. Henriques[3], Sílvia S. Barros[3], Margarida D. Duarte[3]

[1]Instituto de Ciências Agrárias e Ambientais Mediterrânicas (ICAAM), Universidade de Évora, Évora, Portugal; [2]Departamento de Medicina Veterinária, Universidade de Évora, Évora, Portugal; [3]Instituto Nacional de Investigação Agrária e Veterinária (INIAV), Lisboa, Portugal;

Veterinary Microbiology 98:43-50. doi: 10.1016/j.vetmic.2016.12.006

Veterinary Microbiology 198 (2017) 43–50

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Challenges in the rabbit haemorrhagic disease 2 (RHDV2) molecular diagnosis of vaccinated rabbits

C.L. Carvalho[a], E.L. Duarte[a,b], M. Monteiro[c], A. Botelho[c], T. Albuquerque[c], M. Fevereiro[c], A.M. Henriques[c], S.S. Barros[c], Margarida Dias Duarte[c,*]

[a] Instituto de Ciências Agrárias e Ambientais Mediterrânicas (ICAAM), Instituto de Investigação e Formação Avançada (IIFA), Universidade de Évora, Núcleo da Mitra, 7000, Évora, Portugal

[b] Departamento de Medicina Veterinária, Universidade de Évora, Núcleo da Mitra, 7000, Évora, Portugal

[c] Instituto Nacional de Investigação Agrária e Veterinária (INIAV), Av. da República, Quinta do Marquês, 2780-157 Oeiras, Portugal

ARTICLE INFO

Article history:
Received 3 April 2016
Received in revised form 29 November 2016
Accepted 3 December 2016

Keywords:
Rabbit haemorrhagic disease virus
RHDV2
RT-qPCR
RHDV2 vaccines
Vaccinated rabbits
Cq values
Molecular diagnosis

ABSTRACT

Molecular methods are fundamental tools for the diagnosis of viral infections. While interpretation of results is straightforward for unvaccinated animals, where positivity represents ongoing or past infections, the presence of vaccine virus in the tissues of recently vaccinated animals may mislead diagnosis.

In this study, we investigated the interference of RHDV2 vaccination in the results of a RT-qPCR for RHDV2 detection, and possible associations between mean Cq values of five animal groups differing in age, vaccination status and origin (domestic/wild).

Viral sequences from vaccinated rabbits that died of RHDV2 infection (n = 14) were compared with the sequences from the commercial vaccines used in those animals. Group Cq means were compared through Independent *t*-test and One-way ANOVA.

We proved that RHDV2 vaccine-RNA is not detected by the RT-qPCR as early as 15 days post-vaccination, an important fact in assisting results interpretation for diagnosis.

Cq values of vaccinated and non-vaccinated infected domestic adults showed a statistically significant difference ($p < 0.05$), demonstrating that vaccination-induced immunity reduces viral loads and delays disease progression. Contrarily, in vaccinated young rabbits higher viral loads were registered compared to non-vaccinated kittens. No significant variation ($p = 0.3824$) was observed between viral loads of non-vaccinated domestic and wild RHDV2-victimised rabbits. Although the reduced number of vaccinated young animals analysed hampered a robust statistical analysis, this occurrence suggests that passively acquired maternal antibodies may inhibit the active immune response to vaccination, delaying protection and favouring disease progression.

Our finding emphasises the importance of adapting kitten RHDV2 vaccination schedules to circumvent this interference phenomenon.

© 2016 Elsevier B.V. All rights reserved.

1. Introduction

Six years after its emergence in Europe, rabbit haemorrhagic disease virus 2 (RHDV2) continues to provoke severe economic losses in the industry, to cause great concerns on the conservation of diminished wild rabbit populations and dependent endangered carnivore species, and to affect deeply the cinegetic activity and tourism associated income of some countries.

RHDV2, reported for the first time in 2010 (Le Gall-Reculé et al., 2011a), is classified within the *Lagovirus* genus (Le Gall-Reculé et al., 2011a) along with the close genetically related RHDV, European brown hare syndrome virus (EBHSV) and non-pathogentic lagoviruses (Le Gall-Reculé et al., 2011b). Since its emergence in France (Le Gall-Reculé et al., 2011a), RHDV2 quickly spread throughout neighbouring European countries (Dalton et al., 2012; Abrantes et al., 2013; Le Gall-Reculé et al., 2013; Baily et al., 2014; Westcott et al., 2014)(information on the FLI, 10|21|2013), replacing the previously circulating classical strains (Lopes et al., 2015). RHDV2 was registered outside Europe in Australia (Hall et al., 2015).

* Corresponding author.
E-mail address: margarida.duarte@iniav.pt (M.D. Duarte).

http://dx.doi.org/10.1016/j.vetmic.2016.12.006
0378-1135/© 2016 Elsevier B.V. All rights reserved.

Besides the European rabbit, RHDV2 is also able to infect a few hare species (Puggioni et al., 2013; Camarda et al., 2014). The lack of cross protection induced by previous contact with RHDV strains contributed to the rapid spread of RHDV2 in Europe (Le Gall-Reculé et al., 2013), resulting in high mortality rates among naïve wild population soon after its emergence (Delibes-Mateos et al., 2014).

In view of the alarming impact of RHDV2 in the rabbit industry and in wild rabbit populations, and given the urgency in controlling the disease, RHDV2 inactivated vaccines were developed (Filavac VHD Variant, Filavie Laboratories; Cunipravac variant, Hipra; Novarvilap, Ovejero) and provisionally allowed in the European Union member states under special licenses from the Veterinarian Local Authorities. No data is however available about the immunogenicity and success of the inactivated RHDV2 vaccines when applied as a post-exposure tool to infected populations.

Laboratorial confirmation of RHDV2 is required to assist rabbit farms in disease control. Detection of RHDV2 by molecular methods has undeniable advantages due to their unequalled sensitivity and short execution time, allowing a rapid laboratorial response. A specific RT-qPCR with high sensitivity for RHDV2 detection was recently developed (Duarte et al., 2015a), detecting as few as nine molecules of RHDV2 RNA, and has been in use at INIAV Virology Laboratory since 2014.

The interpretation of RT-qPCR results in non-vaccinated animals undergoing acute disease, particularly when low Cq values are obtained, is usually straightforward. Furthermore, RDH characteristic histopathological lesions are generally present, complementing the molecular diagnosis and allowing the confirmation of RHDV2 as the cause of death. Nonetheless, due to the variable mortality rates described for RHDV2 infections (Le Gall-Reculé et al., 2011a, 2013; Dalton et al., 2012), when low viral loads are obtained differential diagnosis is required since positivity may not necessarily relate to clinical state or fatal outcome.

In addition, given the high sensitivity of nucleic acid amplification-based methods, low amounts of vaccine virus in the tissues of RHDV2 vaccinated animals, may compromise the interpretation of the results and the final diagnosis. Interference of inactivated vaccine RNA on real-time RT-PCR results has been investigated for other viruses to assess the potential associations between recent vaccination and RNA detection in blood or tissues. For blue tongue virus (BTV), it was demonstrated that vaccine viral RNA can reach the blood circulation and the spleen in sufficient amounts to be detected by real-time RT-PCR (De Leeuw et al., 2015). Contrarily, previous studies on the RHDV genome persistence in vaccinated rabbits demonstrated that inactivated vaccine RNA was not detected by RT-qPCR, in samples collected nine weeks after vaccination (Gall and Schirrmeier, 2006). However, in that study no evaluation was undertaken for shorter periods after vaccination. With regards to RHDV field strains, genomic RNA or RNA fragments are known to persist in adult rabbits that overcome experimental infection for at least 15 weeks (Gall et al., 2007). Interestingly, in experimentally infected young rabbits, viral RNA was detected as early as 18 h post inoculation in the liver and spleen, but persisted for a shorter period of only 4 weeks (Shien et al., 2000). Antibodies were developed by these young rabbits between 5 and 7 days post inoculation, with titters correlating well with viremia decreased and viral clearance (Shien et al., 2000), reasserting the important role of immune response in disease control.

In this study, we aimed to clarify if RHDV2 vaccines were detected by the RT-qPCR method developed previously (Duarte et al., 2015a) and if the presence of commercial vaccines in the tissues interfered with the detection of field strains RNA. We also investigated the impact of vaccination on the viral loads during infection, by comparing the Cq values from non-vaccinated and vaccinated infected rabbits. For the vaccinated animals, the algorithm routinely followed to achieve a conclusive RHDV2 diagnosis included the differential diagnosis of pathogenic bacteria, classical RHDV and Myxoma virus, to rule out mixed infections, and the screening by RHDV2-RT-qPCR. Histopathology was performed to confirm the presence of characteristic RHD lesions.

2. Materials and methods

2.1. Samples

Cq (quantification cycle) data from a total of 82 animals that died from RHDV2 infection was analysed in the present study. Vaccinated RHDV2-positive domestic rabbits (n = 14) originated in rabbitries from Portugal mainland, where vaccination had been implemented after the laboratorial confirmation of disease in the premises. These samples were obtained during 2015 for the purpose of this study. For the remaining 68 non-vaccinated rabbits, Cq values were obtained under the same laboratorial conditions, while performing diagnosis between 2014 and 2016. Of these, RHDV2-positive liver samples from non-vaccinated domestic rabbits (n = 29) were received at INIAV directly from the veterinarian assistants of industrial rabbitries or through private laboratories. Wild rabbits (n = 39) were found death in hunting and national parks in Portugal mainland and Azores and sent to INIAV for analysis.

Five groups of animals were defined according to age, vaccination status and domestic/wild origin. Young rabbits corresponded to animals with less than 70 days of age. Group 1 included the domestic vaccinated adult rabbits (n = 11). Group 2 comprised domestic young, born from RHDV2 vaccinated does, which were vaccinated before 35 days of age (n = 3), Group 3 encompassed the domestic non-vaccinated adults (n = 23). Group 4 included domestic young, born from RHDV2 vaccinated does, that had not been vaccinated (n = 6). Group 5 comprised the adult, non-vaccinated wild rabbits (n = 39).

Most RHDV2 vaccinated rabbits (>92%) originated in one rabbitry where a mortality of 30%-40% in adults and 80% in the young was registered in the initial outbreak. For one specimen originated in a second farm, no specific information could be obtained apart from the fact that the animals had been vaccinated after disease onset. After the implementation of vaccination, only a few vaccinated adults and non-vaccinated young died sporadically and mortality decreased to 0% in both age groups.

The time that elapsed between the vaccination of animals from Group 1 and 2 and their casualties, varied between 15 and 121 days.

2.2. Vaccines

The identity of the two RHDV2 commercial vaccines used in the 14 vaccinated rabbits (Groups 1 and 2) is not disclosed for ethical and legal reasons. Instead, these vaccines are hereafter referred to as vaccine 1 and vaccine 2. Among the domestic vaccinated adult rabbits (Group 1), 57.14% of the animals were vaccinated with vaccine 1, 14.29% with vaccine 2 and 28.57% with both vaccines. All the vaccinated domestic young rabbits (Group 2) were vaccinated once with vaccine 2.

2.3. Pathological examination

Necropsies were carried out by the veterinarian assistants at the rabbitries or by the pathologists at the Pathology Laboratory of INIAV.

For histopathological examinations, liver and lung samples were fixed in 10% buffered formalin and embedded in paraffin by

standard procedures. Five micrometer-thick sections were stained with haematoxylin and eosin (H&E) and examined using light microscopy (Cook, 1997).

2.4. Bacteriological examination

Liver and lung samples from the 14 vaccinated animals (Groups 1 and 2) were analysed by standard bacteriological culture, including *Pasteurella* sp., which must be considered in the differential diagnosis of RHD according to the OIE (OIE Technical disease cards). Lung and liver samples macerates were inoculated in MacConkey agar (Oxoid) and Colombia agar (Oxoid) supplemented with 5% of defibrinated sheep blood (Biomerieux) and incubated at 37 °C for 24–48 h. Identification of isolates was performed using the commercial API® test strips API 20 NE and API ID32 E (BioMérieux).

None of the non-vaccinated rabbits investigated was submitted to bacteriologic examination.

2.5. RNA extraction and virological examination

Liver and lungs samples were homogenized with phosphate buffered saline (PBS) and clarified at 3000 g for 5 min. DNA and RNA were extracted from 200 μl of the clarified supernatant, corresponding to approximately 50 mg of tissue, in a BioSprint 96 nucleic acid extractor (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Vaccine RNA was extracted from the aqueous phase of a centrifuged sample (10,000*g* for 10 min) of RHDV2 vaccine 2 and of a classical RHDV vaccine (Cylap, Zoetis), used as a negative control, with the RNeasy blood and tissue kit (Qiagen, Hilden, Germany), according to the recommendations. RNA from RHDV2 vaccine 1 was extracted from 200 μl of a 10× diluted sample (v/v in bidistilled H20), in a BioSprint 96 nucleic acid extractor (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Extracted RNA (10 μl) from tissue samples and the three vaccines were assessed by the RT-qPCR developed by (Duarte et al., 2015a). Screening for RHDV (genogroups G1-G6) was performed by sequencing analysis of the amplicons obtained by conventional PCR with primers RC-9 and RC-10 (Tham et al., 1999). Conventional RT-PCR and RT-qPCR were performed using the One Step RT-PCR kit (Qiagen, Hilden, Germany). The presence of myxoma virus was investigated by qPCR (Duarte et al., 2014), using the FastStart TaqMan Probe Master Kit (Roche, Roche Diagnostics GmbH, Manheim, Germany). For the real time PCR systems described, undetectable Cq or Cq values > 40 were considered negative.

2.6. Nucleotide sequencing analysis and alignments

Amplification of the *vp60* sequences of RHDV2 strains and of the two RHDV2 vaccine strains was accomplished with two pairs of primers, 27F (5′-CCATGCCAGACTTGCGTCCC-3′) and 986R (5′-AACCATCTGGAGCAATTTGGG-3′), 717F (5′-CGCAGATCTCCTCA-CAACCC-3′) (Duarte et al., 2015b), and RC10R (Tham et al., 1999) generating two overlapping fragments. The One Step (Qiagen, Hilden, Germany) kit was used, following the recommendations of the manufacturer. Sequencing was carried out using a BigDye™ Terminator cycle sequencing kit (Applied Biosystems, Foster City, CA, USA).

The nucleotide sequences of vaccine and field strains were determined on an automated 3130 Genetic Analyzer system (Applied Biosystems, Foster City, CA, USA).

Nucleotide alignments were performed with Clustal omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) (Sievers et al., 2011).

2.7. Statistical analysis

Descriptive statistics and statistical comparison were performed resourcing to the GraphPad Prism, version 5.00 (GraphPad Software, San Diego California USA, www.graphpad.com), for the Cq values and $\log_{10}$ viral loads, obtained for each sample group.

Absolute quantification was calculated from the equation for the linear regression of the method, assuming that the amount of tissue analysed was the same for all samples (≈50 mg) and that the efficiency of the method was close to 100%.

Statistical comparison of mean Cq value and mean $\log_{10}$ viral loads between two specific groups was carried out using the Independent *t*-test, Welch corrected. To verify the difference in the RT-qPCR results between the animal groups, a One-way ANOVA was performed. A p-value < 0.05 was considered to be statistically significant.

3. Results

3.1. Pathology and microbiology of vaccinated RHDV2-infected rabbits

No signs of disease were registered prior death in the vaccinated animals (n = 14, Groups 1 and 2). However, macroscopic lesions suggestive of haemorrhagic disease were observed in all rabbits, including icteric liver and hepatomegaly, hepatic discoloration, lung petechiae and moderate splenomegaly. At the microscopic level, the lesions matched the typical RHD lesions described before (Ohlinger et al., 1993). Necrotic microfoci in liver parenchyma, hepatocyte hyalinization, severe congestion and disseminated intravascular coagulation (DIC) in the small capillaries were registered. All vaccinated rabbits (Groups 1 and 2) tested negative to RHDV, myxoma virus and *Pasteurella multocida,* and positive to RHDV2, from which they died. Bacteriologic examination was not carried out for any of the non-vaccinated rabbits (Groups 3, 4 and 5), where RHDV2 infection was confirmed as cause of death by the low Cq values obtained and the concomitant presence of RHD typical lesions, regardless of the involvement of other pathogens.

3.2. RT-qPCR detects RNA extracted from two RHDV2 vaccines

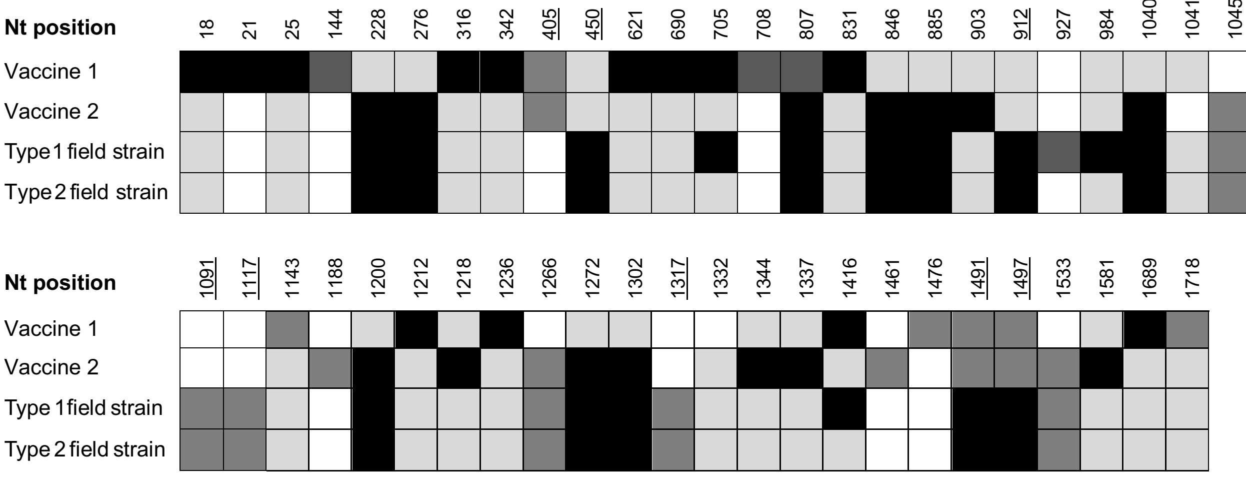
Serial dilutions of RNA from the three vaccines, obtained as described in subsection 3.4, were tested by the RT-qPCR method (Duarte et al., 2015a). In consecutive dilutions of the two RHDV2 vaccines, a Cq value increase of about three folds was registered (*results not shown*). RNA from Cylap (Zoetis), a classical RHDV vaccine, was not detected (*results not shown*).

3.3. The strains characterized from the infected-vaccinated rabbits (Group 1 and group 2) differed from the vaccine strains used

The *vp60* nucleotide sequences of the two RHDV2 vaccines were obtained during this study and compared with sequences amplified from RHDV2 vaccinated victimized rabbits (Group 1 and 2), as well as with field strains sequences obtained in our laboratory and available in public databases.

Vaccine sequences are not disclosed here to ensure that any data that the vaccine companies wish to remain private are not made available. Instead, the comparison of the nucleotide sequences of vaccine and field strains is encoded in Fig. 1. The variability between the two vaccines encompassed 38 residues (Fig. 1), of which only three were non-synonymous (residues at positions 9, 347 and 574).

Among the vaccinated animals' strains characterized in this study, residues at positions 405, 450, 912, 1091 (non-synonymous), 1117, 1317, 1491 and 1497 were found conserved differing from the residues found in the two vaccines (Fig. 1, underlined positions). At



Vaccines 1 and 2 represent the two commercial vaccines analyzed in this study.
Type 1 and 2 field strains represented the two profiles of genetic variability found in the vaccinated rabbits

Fig. 1. Schematic representation of the nucleotide variability found between the two vaccine strains used and two consensus field RHDV2 sequences that represent all the strains obtained from vaccinated animals (bottom two). Underlined residues identify the positions that differ between the two vaccines and the field strains from the vaccinated animal group (Group 1 and Group 2). Each nucleotide is represented by a different colour.

the polypeptide level, one vaccine also differed from the field strains in four residues while the other vaccine only diverged in amino acid 364.

When vaccine strains were compared with the sequences obtained from non-vaccinated animals presently available in our laboratory and in the GenBank, one of them showed to be identical to a field strain collected in 2015 in the South of Portugal (*results not shown*) emphasizing the importance of clarifying if vaccine RNA interferes with the RHDV2 molecular diagnosis. Two single nucleotide polymorphisms, located at nucleotide positions 903 (synonymous) and 1041 (non-synonymous), were identified in the other vaccine, allowing its distinction from all field strains presently known.

3.4. Cq variation among the five groups of animals

To assess the impact of vaccination on disease progression and viral loads, the mean Cq values of vaccinated infected animals were compared with those obtained from non-vaccinated rabbits. Cq values are inversely proportional to the amount of target nucleic acid in the sample, meaning that, lower Cqs correspond to higher viral loads (Bustin et al., 2009).

Domestic vaccinated adults showed lower RNA amounts (Group 1, mean Cq 32.01 $\pm$6.18) than non-vaccinated domestic adults (Group 3) for which a mean Cq value of 15.23 $\pm$ 3.82 was obtained (Table 1). This difference was statistically significant ($p < 0.05$, for a 95% confidence interval (CI), Table 2).

A statistically significant difference was also found between the mean Cq values obtained for vaccinated adults (Group 1) and wild rabbits (Group 5) ($p < 0{,}005$, for a 95% CI, Table 2), for which a mean Cq 14.33 $\pm$ 3.97 was found, meaning high amounts of RNA were present (Table 1).

The difference found between the mean Cq values, of non-vaccinated domestic adults (Group 3) and wild rabbits (Group 5) was not statistically significant ($p = 0.3824$, for a 95% CI, Table 2).

In regard to young rabbits, the viral loads obtained for the vaccinated young (Group 2, mean Cq of 13.80 $\pm$ 2.68) were higher than for the non-vaccinated young rabbits (Group 4, mean Cq of 17.08 $\pm$ 4.17) (Table 1). Nevertheless, this difference was also not statistically significant in *t*-test ($p = 0.2026$, for a 95% CI, Table 2) due to the reduced number of samples.

When vaccinated adult (Group 1) and young rabbits (Group 2) were compared, statistically significant differences in mean Cq values were obtained ($p < 0.05$, for a 95% CI, Table 2). Lower viral loads were found in Group 1 (mean Cq of 32.01 $\pm$6.18) than in Group 2 (mean Cq of 13.80 $\pm$ 2.68) (Table 1).

Basic statistics for the $\log_{10}$ viral loads obtained for the five groups, interpolated from the linear regression curve of the

Table 1
Descriptive statistics analysis of the Cq values and $\log_{10}$ viral charges obtained for the vaccinated and non-vaccinated animal groups' considered in this study. The mean and standard deviation were calculated for both indicators.

		Vaccinated		Non-vaccinated		
		Adult domestic (Group 1)	Young domestic (Group 2)	Adult domestic (Group 3)	Young domestic (Group 4)	Adult wild (Group 5)
Cq values	Sample size (n)	11	3	23	6	39
	Mean	32.01	13.80	15.23	17.08	14.33
	Standard deviation	6.18	2.68	3.82	4.17	3.97
Log_{10} viral Loads	Mean	3.41	8.87	8.49	7.88	8.71
	Standard deviation	1.85	0.80	1.08	1.25	1.21

Table 2
Comparative analysis of mean Cq values and viral loads by unpaired *t*-test analysis, Welch corrected. The means of specific groups were compared in order to address the questions' list displayed.

Question addressed	Compared groups		Sig. (α 0.05)[a]	
			Mean Cq	Mean log_{10} viral loads
What is the impact of vaccination in disease progression in adults?	Group 1 Group 3	Vaccinated domestic adults Non-vaccinated domestic adults	$p < 0.0001^*$	$p < 0.0001^*$
Does age of vaccination affects disease progression?	Group 1 Group 2	Vaccinated domestic adults Vaccinated domestic young	$p < 0.0001^*$	$p < 0.0001^*$
What is the impact of vaccination in disease progression in the young?	Group 2 Group 4	Vaccinated domestic young Non-vaccinated domestic young	$p = 0.2026$	$p = 0.2032$
Does age affects the disease progression in non-vaccinated animals?	Group 3 Group 4	Non-vaccinated domestic adults Non-vaccinated domestic young	$p = 0.3110$	$p = 0.3095$
Is disease progression different in domestic vaccinated and wild rabbits?	Group 1 Group 5	Domestic vaccinated adults Non-vaccinated wild adults	$p < 0.0001^*$	$p < 0.0001^*$
Is disease progression different in domestic non-vaccinated and wild rabbits?	Group 3 Group 5	Non-vaccinated domestic adults Non-vaccinated wild adults	$p = 0.3824$	$p = 0.4674$

Groups 3 and 4 passed the Kolmogorov-Smirnov normality test. Group 2 was not assessed due to the sample size.
*Statistically significant associations for a 95% CI.
[a] Sig (α 0.05)-statistical significance for a Confidence Interval (CI) of 95%.

RT-qPCR method (Duarte et al., 2015a), were also determined and are shown in Table 1. The mean log10 viral loads are represented in Fig. 2.

When performing One-way ANOVA for the comparison of the five groups, differences in the mean Cq values and mean log_{10} viral loads between groups were statistically significant ($p < 0.05$, Table 3).

A concurrent relation between viral load and severity of the microscopic lesions was observed.

4. Discussion

As expected, the RT-qPCR method (Duarte et al., 2015a) developed to detect RHDV2 field strains, also detected efficiently RNA extracted from the two commercial RHDV2 vaccines. Since the strains used by the vaccine manufacturers' are not publicised, their *vp60* gene sequences were decoded during this study (*results not shown*). The analysis showed that one of the vaccines exhibits a single internal mismatch in the reverse primer, already identified in field strains (Duarte et al., 2015b). Given that the method detects any RHDV2 strain, as long as the target region is conserved, sequencing analysis of the complete *vp60* gene was necessary to differentiate vaccine strains from field strains.

Due to the high sensitivity of the molecular method, inactivated vaccine-derived viral RNA could originate weakly positive RT-qPCR results, if still present in the tissues. Several hypotheses have been considered to explain the detection of inactivated vaccine RNA in animal tissues, namely the unintentional intravasal injection of the

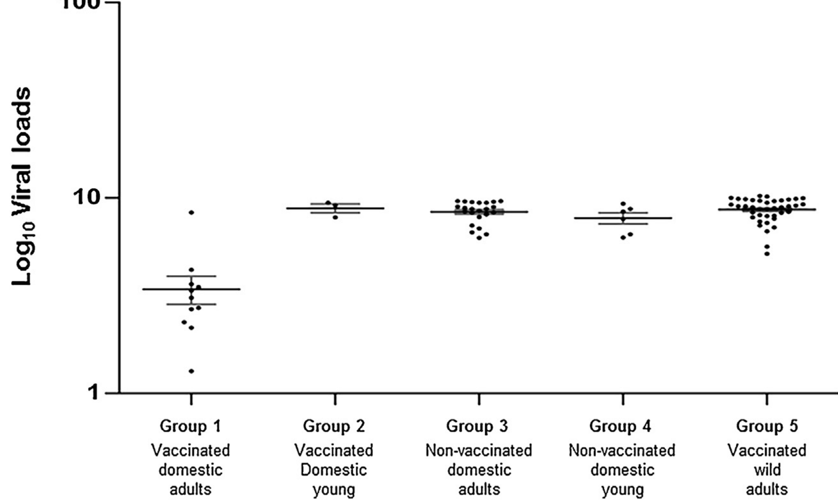


Fig. 2. Log_{10} viral loads obtained for the five groups of animals considered in this study. Dark grey lines and light grey lines show the mean log_{10} viral load and standard deviation calculated for each group.

Table 3
One-way ANOVA comparing the mean Cq values and mean log_{10} viral loads of the five groups of animals specified in this study.

		Sum of squares	df	Mean square	F	Sig (α 0.05)
Cq	Between groups	2897	4	724.30	F (4. 76) = 40.77	$p < 0.0001^{*}$
	Within groups	1350	76	17.77		
	Total	4248	80			
Log_{10} viral loads	Between groups	258.20	4	64.56	F (4. 77) = 40.04	$p < 0.0001^{*}$
	Within groups	124.20	77	1.61		
	Total	382.40	81			

df – degrees of freedom; F – F test; Sig-statistical significance for a Confidence Interval (CI) of 95%.
**p*-value < 0.05 (statistically significant).

vaccine, the enhanced blood permeability at the site of injection, the systemic distribution of viral RNA via phagocytic cells or the association of inactivated virus with erythrocytes (Eschbaumer et al., 2010; Steinrigl et al., 2010; De Leeuw et al., 2015). For instance, Cq values of above 38.1 obtained from cattle blood samples were associated with BTV inactivated vaccine-derived viral RNA (De Leeuw et al., 2015).

For RHDV2, the amount of inactivated vaccine RNA in the different tissues after vaccination was never investigated. However, regarding the closely related RHDV, Gall and collaborators (2006) observed that, a 100% specific multiplex RT-qPCR assay did not detected inactivated-vaccine derived-RNA, nine weeks after vaccination, probably due to the low titres of the virus in each dose (Gall and Schirrmeier, 2006), whereas RHDV viral RNA could be detected for at least 15 weeks after experimental infection (Gall and Schirrmeier, 2006).

In our study, RHDV2 vaccine RNA was never identified in any of the vaccinated animals not even as earlier as 15 days post-vaccination. In fact, sequencing analysis showed that all the strains characterized from vaccinated infected rabbits clearly differed from the ones from the vaccines (Fig. 1).

Possible associations between mean Cq values (or the corresponding viral loads) obtained from rabbits differing in age, vaccination status and origin (domestic/wild) were explored for significant variability. Results showed that the viral loads in vaccinated adults (Group 1, mean Cq value of 32.01 ± 6.18) were much lower than in both non-vaccinated domestic adults (Group 3, mean Cq of 15.23 ± 3.82) and wild rabbits (Group 5, mean Cq of 14.33 ± 3.97). In these two groups viral loads 100,000× and 200,000× higher, respectively, were calculated. For the vaccinated domestic adults a mean viral load of 4.29E + 02 per mg of liver was obtained, about 80,000× lower than the value previously estimated for RHDV2 infected wild rabbits with the same method (1.5×10^{8}copies per mg of tissue) (Duarte et al., 2015b). This reduction in the amount of virus in the liver of vaccinated animals may reflect the effect of vaccination on disease progression and clearly proves its usefulness from clinical and epidemiological points of view. Vaccination is considered an effective post-exposure emergency strategy in farms facing RHD outbreaks (OIE Terrestrial Manual, 2016) since immunity develops rapidly, within seven to 10 days after vaccine administration. Protection conferred by vaccination depends on the dose and on the antibody titre developed. Whenever a protective immune response is produced, vaccination prevents infection and/or clinical signs of disease, depending on antibodies titres (Plotkin, 2008). Regarding RHDV, the inhibitory effects caused by the high level of RHDV antibodies in animals that survived experimental RHDV infection were pointed as a possible reason for the failure of experimental transmission of the virus from a highly immunized rabbit to healthy animals (Gall et al., 2007). Those survivors (five among 50) responded with fever and seroconversion showing high antibody titres, and did not developed further RHD specific symptoms or pathological lesions. The rabbits with the highest viral loads in leukocytes (and also in sera) showed the faster normalization of the body temperature, indicating recovery from disease. The viral load decreased during the experiment (Gall et al., 2007). In our study, the 14 infected/vaccinated rabbits originated from a farm where vaccination was performed when the virus was already circulating. The time at which infection took place regarding vaccination is unknown but the time that elapsed from vaccination and death, ranged between 15 and 121 days. Despite this period was quite variable (2 to 12 weeks), the death of the 14 vaccinated adults suggests that an effective immune response could not be established on time. The exposure to a high infectious dose of field strain, when the vaccine-derived protective immune response was not yet fully established, may have accounted for disease development in these adults, which was confirmed by histopathology.

Higher viral charges were obtained both in non-vaccinated domestic adults (Group 3, mean Cq of 15.05 ± 3.5) and in wild adults (Group 5, mean Cq of 16.31 ± 6.69). The range of viral loads in both groups are close to the values previously described (Duarte et al., 2015b) and suggests that disease progression is similar in domestic and wild rabbits. The highest Cq value in the wild rabbits group (Group 5, upper Cq value 33.6) was significantly above the upper value observed in the non-vaccinated domestic adults group (Group 3, upper Cq value 22.58), probably due to the advanced state of putrefaction of some specimens (n = 4). When these poor quality samples were excluded, the mean Cq value for wild adult rabbits dropped to 14.33 ± 4.0, approaching the homologous value of the non-vaccinated domestic group (mean Cq 15.05 ± 3.5).

Regarding the young rabbits (Groups 2 and 4), a relation between vaccination and reduction of viral amounts was not observed. On the contrary, the viral loads found in vaccinated young rabbits were higher (Group 2, mean Cq 13.80 ± 2.68) than in non-vaccinated kittens (Group 4, mean Cq of 17.08 ± 4.17). In view of the higher mean Cq values observed in non-vaccinated young it is tempting to speculate that a higher antibody response was elicited in these animals where maternal antibodies were not subtracted by vaccine antigens, which suggests that interference with maternal antibodies may impair vaccination success, facilitating disease progression.

Humoral immunity is critical to protect rabbits from RHD (Argüello Villares, 1991; Laurent et al., 1994) and maternal IgG antibodies, acquired during late pregnancy through the placenta and, later on, via colostrum (Lorenzo Fraile, *personal communication*), may be relevant for young rabbits' resistance to RHDV (Cooke, 2002). Rabbit kittens IgGs' can persist for up to 12 weeks after birth (Lengahus C, *unpublished,* cited by (Cooke, 2002)), showing a progressive decline as age and body weight increase (Cooke, 2002). However, maternal antibodies' impact on the RHDV2 vaccination efficacy was never evaluated, but should be taken into account to assure immunization success in RHDV2 vaccination programmes.

Non-vaccinated young, born from RHDV2 vaccinated mothers, showed lower viral loads (Group 4, mean Cq of 17.08 ± 4.17) than non-vaccinated adults (Group 3, mean Cq of 15.05 ± 3.5), in accordance with previous studies (Strive et al., 2010). Although the number of animals available from this group was extremely reduced due to the infrequent fatal outcome in vaccinated animals, this difference may reflect the partial protection conferred by RHDV and RHDV2 immunized mothers. In the RHDV2 infected kittens, a positive association was observed between viral load and the severity of the characteristic anatomopathological lesions found in liver and lungs.

5. Conclusions

One important finding of this study was that, although the RHDV2 RT-qPCR is able to detect vaccine RNA extracted directly from the vaccine suspensions, in animals that had been vaccinated as recently as 15 days before, vaccine RNA did not interfere with the detection of field strains. This piece of information is extremely useful for the overall interpretation of laboratorial results in vaccinated animals, and is especially important as the nucleotide sequences of the vaccines used are usually unknown.

Information on the viral loads is an important addition to qualitative diagnostics. Analysing Cq values obtained in different groups defined according to age, vaccination status, and domestic/wild, allowed us to observe a concurrent relation between Cq values and vaccination in the domestic adults group. Also, no differences in the severity of the disease in domestic and wild rabbits were reported and, accordingly, no significant difference was observed between the viral loads of non-vaccinated domestic and wild rabbits ($p = 0.4674$).

Although the difference was not statistically significant due to sample size, in agreement with (Duarte et al., 2015b), and as it was also described for RHDV (Strive et al., 2010), the present analysis suggest that higher mean viral charges are usually obtained in the non-vaccinated domestic adults than in non-vaccinated young rabbits. Previous studies suggest that proper immune response induced by vaccination may reduce viral titres and the amount of RNA detected by molecular means (Gall and Schirrmeier, 2006). Diagnose based on the detection of low levels of RHDV or RHDV2- RNA should therefore be complemented by histopathology to elucidate infection status. Differential diagnosis with other relevant pathogens should also be considered.

We believe this preliminary investigation provides for the first time, laboratorial data on the effect of post-infection vaccination on molecular diagnosis outcome. The reduced number of samples available from rabbits that died after vaccination (n = 14), constituted the major limitation of this investigation. Although not statistically significant, the trends suggested by the data sets of vaccinated and non-vaccinated young, are in accordance with the notion that early vaccination against RHDV2, similarly to many other viruses such as canine parvovirus (Waner et al., 1996), may be counter-productive due to the presence of the maternal antibodies in the offspring. Vaccination programmes should take into account the inhibitory effect of these antibodies on active immunization that may compromise the success of vaccination of young animals.

Further investigations will have to be conducted on the decay of maternal antibodies and the extent to which they interfere with the active humoral response induced by RHDV2 vaccination in the young, in order to establish more efficient vaccination programmes for the different age groups.

Acknowledgments

The authors thank to Dr. José Monteiro (Coren, SA) and Dr. Carla Afonso and Dr. João Pacheco (Segalab, Laboratório de sanidade animal e segurança alimentar, SA) for providing us most of the samples of vaccinated animals. Our acknowledgements go also to Dr Maria do Carmo Palma Caetano (Serviços de Alimentação e Veterinária da Região do Alentejo), Dr Lucília de Azevedo Mendes (Divisão de Gestão e Autorização de Medicamentos Veterinários, Direção-Geral de Alimentação e Veterinária, Ministério da Agricultura e do Mar), for the commercial vaccines. The authors are also thankful to Maria João Teixeira, Fátima Cordeiro and Ricardino Ferreira for their technical assistance. This study was partially funded by the Fundação para a Ciência e Tecnologia (FCT), grants SFRH/79225/2011 and UID/AGR/00115/2013.

References

Abrantes, J., Lopes, A.M., Dalton, K.P., Melo, P., Correia, J.J., Ramada, M., Alves, P.C., Parra, F., Esteves, P.J., 2013. New variant of rabbit hemorrhagic disease virus, Portugal, 2012–2013. Emerg. Infect. Dis. 19, 1900–1902. doi:http://dx.doi.org/10.3201/eid1911.130908.

Argüello Villares, J.L., 1991. Viral haemorrhagic disease of rabbits: vaccination and immune response. Rev. Sci. Tech. 10, 459–480.

Baily, J.L., Dagleish, M.P., Graham, M., Maley, M., Rocchi, M.S., 2014. RHDV variant 2 presence detected in Scotland. Vet. Rec. 174 doi:http://dx.doi.org/10.1136/vr.g2781 411–411.

Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J., Wittwer, C.T., 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin. Chem. 55, 611–622. doi:http://dx.doi.org/10.1373/clinchem.2008.112797.

Camarda, A., Pugliese, N., Cavadini, P., Circella, E., Capucci, L., Caroli, A., Legretto, M., Mallia, E., Lavazza, A., 2014. Detection of the new emerging rabbit haemorrhagic disease type 2 virus (RHDV2) in Sicily from rabbit (Oryctolagus cuniculus) and Italian hare (Lepus corsicanus). Res. Vet. Sci. 97, 642–645. doi:http://dx.doi.org/10.1016/j.rvsc.2014.10.008.

Cook, H.C., 1997. Origins of tinctorial methods in histology. J. Clin. Pathol. 50, 716–720.

Cooke, B.D., 2002. Rabbit haemorrhagic disease: field epidemiology and the management of wild rabbit populations. Rev. Sci. Tech. 21, 347–358.

Dalton, K.P., Nicieza, I., Balseiro, A., Muguerza, M.A., Rosell, J.M., Casais, R., Álvarez, Á. L., Parra, F., 2012. Variant rabbit hemorrhagic disease virus in young rabbits, Spain. Emerg. Infect. Dis. 18, 2009–2012. doi:http://dx.doi.org/10.3201/eid1812.120341.

De Leeuw, I., Garigliany, M., Bertels, G., Willems, T., Desmecht, D., De Clercq, K., 2015. Bluetongue virus RNA detection by real-time rt-PCR in post-vaccination samples from cattle. Transbound. Emerg. Dis. 62, 157–162. doi:http://dx.doi.org/10.1111/tbed.12100.

Delibes-Mateos, M., Ferreira, C., Carro, F., Escudero, M.A., Gortázar, C., 2014. Ecosystem effects of variant rabbit hemorrhagic disease virus, Iberian Peninsula. Emerg. Infect. Dis. 20, 2166–2168. doi:http://dx.doi.org/10.3201/eid2012.140517.

Duarte, M.D., Barros, S.C., Henriques, A.M., Fagulha, M.T., Ramos, F., Luís, T., Fevereiro, M., 2014. Development and validation of a real time PCR for the detection of myxoma virus based on the diploid gene M000.5L/R. J. Virol. Methods 196, 219–224. doi:http://dx.doi.org/10.1016/j.jviromet.2013.11.014.

Duarte, M.D., Carvalho, C.L., Barros, S.C., Henriques, A.M., Ramos, F., Fagulha, T., Luís, T., Duarte, E.L., Fevereiro, M., 2015a. A real time Taqman RT-PCR for the detection of rabbit hemorrhagic disease virus 2 (RHDV2). J. Virol. Methods 219, 90–95. doi:http://dx.doi.org/10.1016/j.jviromet.2015.03.017.

Duarte, M., Carvalho, C., Bernardo, S., Barros, S.V., Benevides, S., Flor, L., Monteiro, M., Marques, I., Henriques, M., Barros, S.C., Fagulha, T., Ramos, F., Luís, T., Fevereiro, M., 2015b. Rabbit haemorrhagic disease virus 2 (RHDV2) outbreak in Azores: disclosure of common genetic markers and phylogenetic segregation within the European strains. Infect. Genet. Evol. 35, 163–171. doi:http://dx.doi.org/10.1016/j.meegid.2015.08.005.

Eschbaumer, M., Wäckerlin, R., Rudolf, M., Keller, M., König, P., Zemke, J., Hoffmann, B., Beer, M., 2010. Infectious blood or culture-grown virus: a comparison of bluetongue virus challenge models. Vet. Microbiol. 146, 150–154. doi:http://dx.doi.org/10.1016/j.vetmic.2010.05.004.

Gall, A., Schirrmeier, H., 2006. Persistence of rabbit haemorrhagic disease virus genome in vaccinated rabbits after experimental infection. J. Vet. Med. B Infect. Dis. Vet. Public Health 53, 358–362. doi:http://dx.doi.org/10.1111/j.1439-0450.2006.00986.x.

Gall, A., Hoffmann, B., Teifke, J.P., Lange, B., Schirrmeier, H., 2007. Persistence of viral RNA in rabbits which overcome an experimental RHDV infection detected by a highly sensitive multiplex real-time RT-PCR. Vet. Microbiol. 120, 17–32. doi:http://dx.doi.org/10.1016/j.vetmic.2006.10.006.

Hall, R.N., Mahar, J.E., Haboury, S., Stevens, V., Holmes, E.C., Strive, T., 2015. Emerging rabbit hemorrhagic disease virus 2 (RHDVb). Aust. Emerg. Infect. Dis. 21, 2276–2278. doi:http://dx.doi.org/10.3201/eid2112.151210.

Laurent, S., Vautherot, J.F., Madelaine, M.F., Le Gall, G., Rasschaert, D., 1994. Recombinant rabbit hemorrhagic disease virus capsid protein expressed in baculovirus self-assembles into viruslike particles and induces protection. J. Virol. 68, 6794–6798.

Le Gall-Reculé, G., Zwingelstein, F., Boucher, S., Le Normand, B., Plassiart, G., Portejoie, Y., Decors, A., Bertagnoli, S., Guérin, J.-L., Marchandeau, S., 2011a. Detection of a new variant of rabbit haemorrhagic disease virus in France. Vet. Rec. 168, 137–138. doi:http://dx.doi.org/10.1136/vr.d697.

Le Gall-Reculé, G., Zwingelstein, F., Fages, M.-P., Bertagnoli, S., Gelfi, J., Aubineau, J., Roobrouck, A., Botti, G., Lavazza, A., Marchandeau, S., 2011b. Characterisation of a non-pathogenic and non-protective infectious rabbit lagovirus related to RHDV. Virology 410, 395–402. doi:http://dx.doi.org/10.1016/j.virol.2010.12.001.

Le Gall-Reculé, G., Lavazza, A., Marchandeau, S., Bertagnoli, S., Zwingelstein, F., Cavadini, P., Martinelli, N., Lombardi, G., Guérin, J.-L., Lemaitre, E., Decors, A., Boucher, S., Le Normand, B., Capucci, L., 2013. Emergence of a new lagovirus related to rabbit haemorrhagic disease virus. Vet. Res. 44, 81. doi:http://dx.doi.org/10.1186/1297-9716-44-81.

Lopes, A.M., Correia, J., Abrantes, J., Melo, P., Ramada, M., Magalhães, M.J., Alves, P.C., Esteves, P.J., 2015. Is the new variant RHDV replacing genogroup 1 in Portuguese wild rabbit populations? Viruses 7, 27–36. doi:http://dx.doi.org/10.3390/v7010027.

OIE Terrestrial Manual, 2016. Chapter 2 6.2. Rabbit Haemorrhagic Disease. .

Ohlinger, V.F., Haas, B., Thiel, H.J., 1993. Rabbit hemorrhagic disease (RHD): characterization of the causative calicivirus. Vet. Res. 24, 103–116.

Plotkin, S.A., 2008. Vaccines: correlates of vaccine-induced immunity. Clin. Infect. Dis. 47, 401–409. doi:http://dx.doi.org/10.1086/589862.

Puggioni, G., Cavadini, P., Maestrale, C., Scivoli, R., Botti, G., Ligios, C., Le Gall-Reculé, G., Lavazza, G., Capucci, A., 2013. The new french 2010 rabbit hemorrhagic disease virus causes an RHD-like disease in the sardinian cape hare (Lepus capensis mediterraneus). Vet. Res. 44, 96. doi:http://dx.doi.org/10.1186/1297-9716-44-96.

Shien, J.H., Shieh, H.K., Lee, L.H., 2000. Experimental infections of rabbits with rabbit haemorrhagic disease virus monitored by polymerase chain reaction. Res. Vet. Sci. 68, 255–259. doi:http://dx.doi.org/10.1053/rvsc.1999.0372.

Sievers, F., Wilm, A., Dineen, D., Gibson, T.J., Karplus, K., Li, W., Lopez, R., McWilliam, H., Remmert, M., Söding, J., Thompson, J.D., Higgins, D.G., 2011. Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. Mol. Syst. Biol. 7, 539. doi:http://dx.doi.org/10.1038/msb.2011.75.

Steinrigl, A., Revilla-Fernández, S., Eichinger, M., Koefer, J., Winter, P., 2010. Bluetongue virus RNA detection by RT-qPCR in blood samples of sheep vaccinated with a commercially available inactivated BTV-8 vaccine. Vaccine 28, 5573–5581. doi:http://dx.doi.org/10.1016/j.vaccine.2010.06.034.

Strive, T., Wright, J., Kovaliski, J., Botti, G., Capucci, L., 2010. The non-pathogenic Australian lagovirus RCV-A1 causes a prolonged infection and elicits partial cross-protection to rabbit haemorrhagic disease virus. Virology 398, 125–134. doi:http://dx.doi.org/10.1016/j.virol.2009.11.045.

Tham, K.M., Barnes, S.M., Hunter, S.N., 1999. Polymerase chain reaction amplification and gene sequence analysis of a calicivirus from a feral rabbit. Virus Genes 18, 235–242.

Waner, T., Naveh, A., Wudovsky, I., Carmichael, L.E., 1996. Assessment of maternal antibody decay and response to canine parvovirus vaccination using a clinic-based enzyme-linked immunosorbent assay. J. Vet. Diagn. Invest. 8, 427–432.

Westcott, D.G., Frossard, J.-P., Everest, D., Dastjerdi, A., Duff, J.P., Choudhury, B., 2014. Incursion of RHDV2-like variant in great britain. Vet. Rec. 174 doi:http://dx.doi.org/10.1136/vr.g2345 333–333.

Chapter II

World Organisation for Animal Health (OIE) information on RHD sanitary status and importation recommendations

1. Review on the OIE information on sanitary status and importation recommendations on RHD

1.1. Notification of animal diseases

The successful control of a disease, and a potential epidemic, depends on rapid access to complete information on the national disease situation (Vallat et al., 2013). Diseases must be immediately notified in a clear and transparent way to ensure a timely response (Vallat et al., 2013). Disease notification may have a negative impact on the economic performance of a country by causing loss of export markets or discouraging tourism. A countries' credibility in terms of disease reporting is based on timely and accurate notification of diseases (Vallat et al., 2013).

RHD integrates the OIE list of notifiable terrestrial and aquatic animal diseases, which in 2016 replaced the former Lists A and B (OIE). The former List A integrated transmissible diseases with potential for very serious and rapid spread, irrespective of national borders, serious socio-economic or public health consequence and of major importance in the international trade of animals and animal products (OIE). The previous List B, included transmissible diseases considered to be of socio-economic and/or public health importance within countries and significant in the international trade of animals and animal products. RHD integrated List B in the past.

The OIE defined criteria to examine the inclusion or not of a given disease in the OIE single list were approved in May 2004 (OIE). The list is reviewed on a regular basis and when there are modifications adopted by the World Assembly of Delegates at its annual General Session, the new list comes into force on 1st January of the following year (OIE). The information on RHD sanitary status and importation recommendations figuring in the OIE terrestrial animals' sanitary code is presented in **Annex III.**

As the OIE official notifications are sporadic and given the urgency and relevance in the release of important information to the scientific community but also to various layers of the society, including conservationists, veterinaries, hunters, hunting tourism agents as well as the general public, disclosure of such important information is often communicated in oral communications or posters in congresses, lectures or even in letters to the editor. This was the case of the report made by our team in early 2015 informing on the RHDV2 detection in domestic rabbits on the Azorean archipelago, only a few months after the disease was first reported in the wild rabbit populations (**Study 4**).

Study 4

What is the importance of early disease report?

Detection of rabbit haemorrhagic disease virus 2 (RHDV2) in domestic rabbits in Azores

Margarida D. Duarte[1], Ana M. Henriques[1], Sílvia S. Barros[1], Teresa Fagulha[1], Fernanda Ramos[1], Tiago Luís[1], Miguel Fevereiro[1], Carina L. Carvalho[2], Sandra Benevides[3], Lídia Flor[3], Sílvia V. Barros[3], Susana Bernardo[3]

[1]Instituto Nacional de Investigação Agrária e Veterinária (INIAV), Lisboa, Portugal; [2]Instituto de Ciências Agrárias e Ambientais Mediterrânicas (ICAAM), Universidade de Évora, Évora, Portugal; [3]Laboratório Regional de Veterinária, Açores, Portugal

Veterinary Record 2015 176: 499-500

Downloaded from http://veterinaryrecord.bmj.com/ on May 8, 2015 - Published by group.bmj.com

RABBIT HEALTH

Detection of RHDV variant 2 in domestic rabbits in Azores

FOLLOWING on from our letter published earlier this year (*VR*, January 31, 2015, vol 176, p 130), which reported the detection of rabbit haemorrhagic disease virus variant 2 (RHDV-2) in wild rabbits from four islands of the western and central group of the Azorean archipelago, we would like to communicate the diagnosis of RHDV-2 in domestic rabbits from Terceira Island.

Two adult rabbits were diagnosed with RHDV-2 (Duarte and others 2015). They originated from the localities of Quatro-Ribeiras and São Bento, located on the north and south coasts of Terceira Island. The deaths occurred on January 28 and February 23, 2015, respectively. The animals were kept in small backyard rabbitries. There were seven rabbits kept at Quatro-Ribeiras (Fig 1) and 13 were kept at São Bento. None had been vaccinated against RHDV or RHDV-2.

Pathological findings showed congestion

Downloaded from http://veterinaryrecord.bmj.com/ on May 8, 2015 - Published by group.bmj.com

FIG 1: Rabbitry in Quatro-Ribeiras on the north coast of Terceira Island, Azores

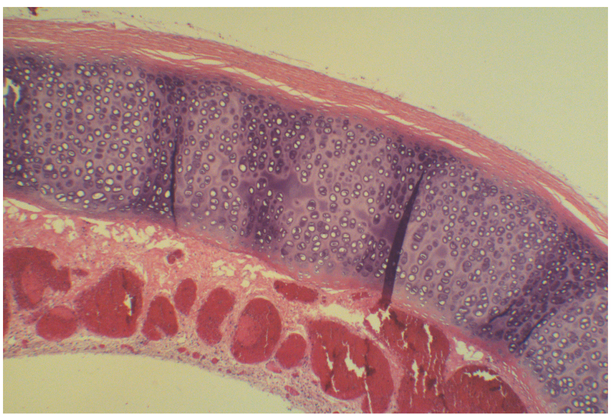


FIG 2: Histopathological examination of the trachea of a RHDV-2-positive domestic rabbit from São Bento, showing intense congestion in the mucosa

and haemorrhage in the lungs and tracheal mucosa (Fig 2), and discoloration of the liver compatible with RHDV.

Sequencing analysis of the *vp60* gene revealed that both RHDV-2 strains were highly similar, differing only in two synonymous substitutions. The single nucleotide polymorphisms previously identified in wild rabbit RHDV-2 strains from Flores, Graciosa, São Jorge and Terceira islands discussed in our previous letter, were also present in these two strains, suggesting the wild populations as the source of infection.

A retrospective inquiry revealed that two other animals, one from each rabbitry, had also possibly contracted RHDV-2, although these suspicions were not confirmed by laboratory diagnosis. There were no other occurrences after the death of the two animals reported here. Interestingly, a dead wild rabbit had recently been found in close proximity to the rabbitry at Quatro-Ribeiras. In this case, since the owner fed the animals with naturally occurring local vegetation, transmission may have occurred through direct contact with RHDV-2 contaminated plants.

In the São Bento case, the cages were not in direct contact with the surrounding grassland fields and the animals were fed exclusively with dry feed. However, the owner reported the presence of rats in the area, raising questions about their potential role in the transmission of RHDV-2 from the wild rabbit populations. Also, viral contamination of fomites may have occurred since the owner works in agriculture and farms extensively. Although mechanical vectors such as mosquitoes and flies are less active during this time of year, their hypothetical role in RHDV-2 transmission from wild to domestic rabbits needs to be considered.

In addition, since our previous report, we have confirmed the presence of RHDV-2 in wild rabbits originated from Pico. With exception of Corvo Island, which is not inhabited by wild rabbits, RHDV-2 has spread through the entire Azorean archipelago.

Margarida Duarte, **Margarida Henriques**, **Silvia C. Barros**, **Teresa Fagulha**, **Fernanda Ramos**, **Tiago Luís**,**Miguel Fevereiro**, National Institute of Agrarian and Veterinarian Research, Virology Laboratory, Rua General Morais Sarmento, 1500-310 Lisbon, Portugal
e-mail: margarida.duarte@iniav.pt
Carina Varvalho, University of Évora, ICAAM/Science and Technology School, Apartado 92, 7002-554 Évora, Portugal
Sandra Benevides, **Lídia Flor**, **Sílvia Vanessa Barros**, **Susana Bernardo**, Veterinary Regional Laboratory of Angra do Heroísmo, Vinha Brava 9700- 236, Angra do Heroísmo, Azores, Portugal

Reference

DUARTE, M. D., CARVALHO, C. L., BARROSA, S. C., HENRIQUES, A. M., RAMOS, F., FAGULHA, T. & OTHERS (2015) A real time Taqman RT-PCR for the detection of rabbit hemorrhagic disease virus 2 (RHDV2). *Journal of Virological Methods* 219, 90-95

doi: 10.1136/vr.h2402

Chapter III

RHD pathogenesis and prophylaxis

1. Review on RHD pathogenesis

Rabbit haemorrhagic disease (RHD) is a highly contagious, severe acute viral disease of wild and domestic rabbits that has been responsible worldwide for a significant decline in the number of wild rabbits (Ferreira et al., 2005)(Abrantes et al., 2013)(Delibes-Mateos et al., 2014) and as caused important losses in the rabbit industry (McIntosh et al., 2007)(Le Gall-Reculé et al, 2011a)(Dalton et al., 2012).

1.1 Classical rabbit haemorrhagic disease virus (RHDV)

The disease caused by the RHDV is characterised by high morbidity and mortality, which ranges from 70% to 95% in adult animals (McIntosh et al., 2007). The virus replicates in the liver inducing a fulminant hepatitis and disseminated intravascular coagulation (DIC) that leads to death (Marcato et al., 1991)(Mikami et al., 1999).

Rabbits up to 4 weeks of age are usually resistant, do not exhibit clinical signs and survive infection (Ohlinger et al., 1993)(Mikami et al., 1999)(Ferreira et al., 2005)(McIntosh et al., 2007).

Marcato et al. (1991) reviewed the spectrum of clinical responses to RHDV, and referred five forms of the disease. The peracute form, affecting highly susceptible rabbits not previously infected that die suddenly without any clinical sign, although haematuria and/or vaginal haemorrhage and epistaxis are occasionally reported. The acute form affects adult or young rabbits under 2-months old and is highly prevalent in epidemic areas. The subacute form, occurring with attenuated symptoms in the later stages of an epidemic; most animals survive becoming resistant to reinfection. The chronic form is infrequent and symptomless and the subclinical form is only assumed in suckling rabbits.

1.1.1. Adult rabbits

1.1.1.1. Clinical symptoms

The incubation period of acute disease is usually short ranging from 1 to 2 days and occasionally 3 days (Argüello-Villares et al., 1988)(Marcato et al., 1991). Typically, diseased animals present fever (>40°C), and death may occur rapidly within 12 to 48 hours after exposure (Argüello-Villares et al., 1988)(Capucci et al., 1991)(Marcato et al., 1991)(McIntosh et al., 2007). Shortly before death the animals may show hypothermia (Ohlinger et al., 1993). Anorexia, apathy and tachypnoea have also been reported (Ohlinger et al., 1993)(McIntosh et al., 2007) and the animals often develop severe dyspnoea and neurologic signs including ataxia and convulsions preceding death (Argüello-Villares

et al., 1988)(Marcato et al., 1991)(McIntosh et al., 2007). Epistaxis is observed in dead animals (Argüello-Villares et al., 1988).

Subacute cases (5% to 10% of infected rabbits) may present with malaise, mild anorexia, apathy, weight-loss and jaundice (Marcato et al., 1991)(Ohlinger et al., 1993)(McIntosh et al., 2007). Death may occur within 1 to 2 weeks after infection (Marcato et al., 1991)(Ohlinger et al., 1993)(McIntosh et al., 2007).

Rabbits under 45-50 days of age are usually resistant and survive infection without the presentation of clinical signs (McIntosh et al., 2007)

1.1.1.2. Pathological lesions

1.1.1.2.1. Macroscopic lesions

At necropsy, rabbits often present epistaxis (Marcato et al., 1991) and generalized congestion of all organs (Argüello-Villares et al., 1988). As a rule, the blood coagulation is significantly inhibited (Marcato et al., 1991)(Ohlinger et al., 1993). The most severe lesions are found in the liver, trachea and lungs (Marcato et al., 1991) but the kidney and spleen are also affected (Ohlinger et al., 1993).

Hydrothorax is observed in some cases (Ohlinger et al., 1993). Lungs show varying degrees of haemorrhagic lesions, including petechiae (Ohlinger et al., 1993)(Argüello-Villares et al., 1988). Bloody foam is usually observed in the bronchia and trachea that is typically severely haemorrhagic (Marcato et al., 1991)(Ohlinger et al., 1993)(Argüello-Villares et al., 1988).

A generalized congestion of the intestines can be observed as well as a sero-haemorrhagic fluid and the liver is usually enlarged, friable and congested (Argüello-Villares et al., 1988)(Ohlinger et al., 1993). This organ may present pale yellow, greyish or dark reddish discoloured (Marcato et al., 1991)(Argüello-Villares et al., 1988)(Ohlinger et al., 1993). Accentuation of the lobular markings is observed. The spleen is often normal in its appearance but splenomegaly has also been reported. The kidneys are congested and enlarged with sub-capsular lesions including ecchymosis and haemorrhages (Argüello-Villares et al., 1989). They are soft and the renal capsule is easily detached. There are haemorrhages in the cortical and medullar areas of the kidney. In the central nervous system, congestion and haemorrhages are perceived (Argüello-Villares et al., 1988).

Foetus of pregnant does may present multi-focal haemorrhages (Marcato et al., 1991).

1.1.1.2.2. Microscopic lesions

1.1.1.2.2.1. Liver

Microscopic lesions are very marked in the liver and lungs. The liver is the most severely affected organ with an acute necrotic hepatitis, multifocal necrosis and early leukocytic infiltration (Marcato et al., 1991)(Mikami et al., 1999). The foci may become confluent forming extensive local areas, mainly at the lobules periphery. Intra-sinusoidal micro-thrombi maybe present inside small necrotic foci (Marcato et al., 1991). These strong degenerative and necrotic alterations have major diagnostic significance.

Other hepatocytic lesions include hydropic rarefaction and cytoplasmic swelling, microvascular steatosis, apoptosis, bile pigment and/or iron pigment deposition, megalocytosis of single hepatocytes, binucleation and dystrophic granular calcification. The mild to moderate inflammatory infiltrate consists of lymphocytes in portal spaces and sinusoids, and granulocytes in sinusoids (only in foci of necrosis). As an infrequent finding, a moderate periportal fibrosis can be observed (Marcato et al., 1991).

In early stages of infection, virus-like electron dense particles were detected intra-nuclearly and later on within the cytoplasm and similar particles were also found in endothelial cells (Ohlinger et al., 1993).

1.1.1.2.2.2. Lungs and trachea

Tracheal and pulmonary lesions are mainly of the hyperaemic-oedematous type (Marcato et al., 1991). Histopathologically, in the lungs alveolar oedema, haemorrhages and infiltrates of granulocytes were detected as well as microthrombi in alveolar capillaries. Congestion of the tracheal sub-mucosal capillaries, leukocyte infiltration and calcification of the tracheal cartilage have also been registered (Marcato et al., 1991)(Ohlinger et al., 1993).

1.1.1.2.2.3. Other organs

Karyorrhexis of lymphoid tissue can lead to lymphocytopaenia and leukocytopaenia (Huang, 1991)(Marcato et al., 1991). Marques et al. (2010) showed that RHDV induces an early decrease in B and T cells in both spleen and liver before the animals show enzymatic evidence of liver damage. This depletion persisted after liver transaminases increment. It was suggested that these alterations may precede or attend liver damage.

Glomerular hyalinic thrombosis, dilated tubules and lymphocytic infiltrates and epithelial calcification are other reported lesions in the kidneys (Marcato et al., 1991)(Ohlinger et al., 1993). The spleen may show hyperaemia with singular follicular karyorrhexis (Ohlinger et al., 1993).

In the central nervous system microthrombosis is rarely found and other lesions may include intramyelinic oedema (Marcato et al., 1991).

Fibrinous thrombi are observed within the capillaries of most organs. Thrombocytopenia and prolonged prothrombin- and thrombin-times indicate DIC that can result either from endothelial destruction or necrosis of the liver (Xu and Chen, 1989)(Marcato et al, 1991)(Ohlinger et al., 1993).

1.1.1.3. Pathogenesis

The oral route is the main route of infection of RHD, followed by the conjunctival and respiratory routes and skin trauma (Marcato et al., 1991).

Liver damage plays a key role in the pathogenesis of RHD since the animals die from fulminant hepatitis (Mitro and Krauss, 1993)(Marcato et al., 1991)(Marques et al., 2010). Likewise, the directly virus-induced endothelial injury may also contribute to the rapid course of the disease by initiating DIC and/or haemorrhages (Marcato et al., 1991). In alternative, DIC may also result of massive hepatic necrosis leading to activation of extrinsic factors and failure of clearance of activated pro-coagulant factors (Marcato et al., 1988).

Fulminant hepatitis and DIC may not be the only mechanism in the pathogenesis of RHD, as proposed by Marques et al. (2010) that suggest that leukopenia may contribute to RHDV pathogenesis. Humoral immunity is critical to protection from RHD (Argüello-Villares et al., 1991)(Huang et al., 1991)(McIntosh et al., 2007) and, as the immune system is severely affected and animals usually die in 1 to 3 days, infected rabbits are unable to mount a specific and effective immune response against the virus (Huang et al., 1991)(Marques et al., 2014).

Ferreira et al. (2005) suggest that the innate immune response against RHDV may differ in young and adult rabbits, as the cellular inflammatory response of the liver to RHDV is different in susceptible adult rabbits and in resistant young rabbits. In adult rabbits, liver infiltrates were mostly composed of heterophils located near hepatocytes, showing severe cellular damage. In this case, leukocyte infiltration is probably directed at the removal of dead hepatocytes. In contrast, liver leukocyte infiltrates of RHD-resistant young rabbits included predominantly lymphocytes showing membrane contacts with the undamaged hepatocytes cell surface, suggesting molecular changes on the hepatocytes cell surface namely the expression of viral antigens. In addition, when

investigating the role of immunity in the resistance of young rabbits to RHDV, Marques et al. (2014) verified if immunosuppression would change the virus progression. These researchers found that young rabbits under immunosuppression induced by a long-term corticosteroid presented depletion of both T and B cells and died from RHDV infection within 3 days, similarly to adult rabbits.

In the initial stages of RHDV infection, specific immunity results from coordinated interaction of T and B lymphocytes (Huang et al., 1991). According to Li (1990) and Huang et al. (1991) high titres of interferon (IFN) can interfere with RHDV pathogenesis and provide non-specific protection, suggesting its important role in the immune response to RHDV. Rabbits showing high titres of IFN seem to recover from RHD and it is also suggested that IFN contributes to the initial immune responses post vaccination (Huang et al., 1991).

1.1.2. Young rabbits

Despite the absence of clinical signs in young rabbits, Ferreira et al. (2004) documented that the infection causes a transient disease in these animals with enhancement of liver transaminases and decrease in blood heterophils. In addition, Mikami et al. (1999) described the hepatic lesions in young rabbits with 2 weeks and 4 weeks old experimentally infected with RHDV. In that study, hepatic lesions were observed at 12 hours post-infection (PI) in 2 week-old rabbits and at 24 hours PI in 4 week-old ones. Only a small number of hepatocytes was infected by RHDV. The histopathological lesions were more severe in the 4-week old rabbits, suggesting that these animals are more susceptible to RHDV. The hepatic lesions found in young rabbits comprised isolated hepatic foci of necrosis and scattered cellular aggregates including degenerated hepatocytes. Acidophilic or Councilman bodies, recognized as apoptotic of hepatocytes, were also observed. Cellular aggregates were considered a reaction to the hepatocyte necrosis.

The fact that in the Mikami et al. (1999) study, the animals did not developed clinical disease supported the epidemiological evidence that rabbits less than 2 months old do not develop clinical disease. Although resistant to RHD, infected young rabbits may act as long-term carriers RHDV, representing a major source of virus transmission (Ferreira et al., 2004)(Matthaei et al., 2014) and may play an important role in RHDV epidemiology, in particular for virus transmission within social groups during outbreaks (Matthaei et al., 2014).

1.1.2.1. Mechanisms of resistance of young rabbits to RHDV

Young rabbits up to 4-weeks of age are naturally resistant to classical RHDV (Liu et al., 1984) However, when kits older 4-weeks of age are infected, mortality increases to reach the rates of adult rabbits at about 9 weeks of age (Morrise et al., 1991)(Ruvoën-Clouet et al., 1995). The tracheal and duodenal epithelial cells of young rabbits were shown to be nearly devoid of A and H type 2 antigens, reducing RHDV binding (Ruvoën-Clouet et al., 1995)(Ruvoën-Clouet et al., 2000). In accordance, the age-dependent expression of H type 2 correlates with an increase in susceptibility to RHDV infection (Ruvoën-Clouet et al., 2000).

Since the liver does not express HBGAs, it is hypothesized that alternative RHDV cellular receptors must be present, as large numbers of RHDV VLPs are detected in the liver of infected rabbits (Ruvoën-Clouet et al., 2000). In accordance, hepatic lesions in young rabbits suggest that RHDV resistance mechanisms go beyond the virus attachment to host cells HBGAs (Mikami et al., 1999). An hypothesis is that resistance of young rabbits to RHDV is related to changes in liver function (Morrise et al., 1991)(Mikami et al., 1999). Rabbits are mostly resistant to RHDV infection during the weaning period (Liu et al., 1984) and feeding change from milk to grass could alter liver function, resulting in a higher susceptibility to RHDV (Mikami et al., 1999). The structure and function of the liver changes with age; differentiation and cell maturation influences susceptibility to infection and ability to support viruses' replication (Mims, 1989).

Natural resistance of young rabbits to RHDV infection is associated with a rapid and effective inflammatory response of the liver, with few hepatocytes infected, and with a sustained elevation of local and systemic B and T cells (Marques et al., 2012). In young rabbits, RHDV infection was associated with the rise of pro-inflammatory cytokines including the TNF-α, IL-1, IFN-α, IFN-γ, IL-6 and IL-8 as early as 6 hours PI (Marques et al., 2012).

1.1.3. Resistance mechanisms to RHDV

1.1.3.1. HBGA polymorphisms

HBGA diversity in rabbits duodenum its' thought to restrict the virus transmission and generate genetic resistance to RHDV at the population level (Guillon et al., 2009)(Nyström et al., 2011), suggesting a co-evolution between RHDV and its' rabbit host (Harrington et al. 2002)(Marionneau et al. 2002). RHDV have possibly exerted a very strong selective pressure on its host selecting positively animals with diminished expression of H type 2 (Guillon et al., 2009), favouring a

decrease in the virus transmission and mutant strains with lower virulence (Fouchet et al., 2009)(Guillon et al., 2009).

Classical RHDV G1 to G6 proven to bind to HBGAs in a strain-dependent manner and with variable magnitudes depending on their HBGA binding characteristics (Nyström et al., 2011). The G6 VLP pseudoatomic model determined by Wang et al., (2013) enabled the identification of seven regions of sequence variation on the P domain, which could produce different HBGA binding specificities and antigenicity. Hence, three putative HBGAs binding cavities were anticipated on the G6 P domain outer surface (Wang et al., 2013).

All classical RHDV strains were shown to bind H and B type 2 HBGAs but A type was not recognized by G2 or G3, and Lewis Y was only recognized by G1 and G6 (Leuthold et al., 2015). RHDV strains were not A, B or H specific, but rather relatively dependent on the level of expression of each one of these antigens (Nyström et al, 2011). However, B antigen appeared to have a greater importance over A when both were present, even when A antigen expression was apparently higher in the duodenum. Classical RHDV was shown to bind strongly to B antigen than to A antigen. Also, B antigen is always present when A antigen is expressed but A antigen can be expressed in absence of B (A+B- animals) (Nyström et al, 2011). Although infection with a high viral dose can compensate for weak viral binding to HBGAs, as demonstrated by Nyström et al. (2011), untypically high survival rates were recorded with lower virus loads in the survivors' tissues. The same authors conducted a series of experiments proving that, at low viral titres, adult rabbits expressing low amounts of HGBAs were less susceptible to RHDV that those expressing high amounts, even though all animals were infected.

1.1.3.1.1. Genes encoding the α1,2-fucosyltransferase implicated in the synthesis of H type 2

The *Fut1*, *Fut2*, and *Sec1* genes, encoding the α1,2-fucosyltransferase implicated in the synthesis of H type 2, are thought to be located nearby in the rabbit genome (Guillon et al., 2009). However, the Guillon et al. (2009) study showed that while *Fut1* presented almost no variation, *Fut2* showed a high number of mutations and *Sec1* even a higher number. In their study aiming to evaluate an association between expression of the H histo-blood group antigen, α1,2fucosyltransferases polymorphism of wild rabbits, and sensitivity to rabbit haemorrhagic disease virus, Guillon et al. (2009) found that *Sec1v5* allele was strongly associated with RHD resistance, as its frequency was significantly higher among survivors than non-survivors. However, this is not a null allele and it is always associated with a functional *Fut2*, compared to which it has lower activity, hence

constituting a marker of survival but not the origin of the nonsecretor-like phenotype related with RHDV resistance (Guillon et al., 2009). These authors hypothesized that the *Sec1v5* allele was probably genetically linked with a mutation located in the *Fut2* gene compromising *Fut2* enzymes and the production of the virus ligand.

1.2. Rabbit haemorrhagic disease virus 2 (RHDV2)

As demonstrated by Le Gall-Reculé et al. (2013) under experimental conditions, RHDV2 is less virulent than RHDV as mortalities occur later and over a longer period. The disease developed also differs from that induced by RHDV in its clinical presentation, given that chronic and sub-acute forms are more frequent in RHDV2 infections (Le Gall-Reculé et al., 2013). Macroscopic and histopathological lesions are typical and similar to RHDV infection (Le Gall-Reculé et al., 2013)(Lopes et al., 2015a) as previously described. Nevertheless, a higher frequency of severe liver degeneration, splenomegaly and jaundice are seen, characteristic of the subacute/chronic form of RHD (Le Gall-Reculé et al., 2013).

Rabbit kits as young as 11 days old, are susceptible to RHDV2 and develop the disease (Le Gall-Reculé et al., 2011a)(Dalton et al., 2012)(Le Gall-Reculé et al., 2013). In infected kits, macroscopic lesions are consistent with the RHD lesions infection observed in adult rabbits (Dalton et al., 2012).

Mortality rates of up to 20% and 50% in adult and young rabbits, respectively, were described by Dalton et al. (2012).

There is still limited information on RHDV2 interactions with HBGAs or other receptors that could explain differences in RHDV2 pathogenesis. When G6 and RHDV2 unbound H2-tri structures were super-positioned, a similar HBGA binding pocket was observed (Leuthold et al., 2015). HBGA binding residues were shown to be preserved among RHDV strains, indicating that various strains bind HBGA at the same pocket (Leuthold et al., 2015). In addition, the flexibility found in G6 and RHDV2 HBGA binding pocket may protect it from the immune system while promoting HBGA binding diversity among different RHDV strains (Dalton et al., 2012)(Leuthold et al., 2015).

When the RHDV2 P domain in complex with Lewis Y and H type 2 HBGA was investigated, these attachment factors were shown to bound at the dimeric interface on the side of the RHDV2 P dimer, clearly differing from the three G6 P domain predicted sites (Wang et al., 2013)(Leuthold et al., 2015).

The macro and microscopic lesions induced by RHDV2 have also been described in this work, namely in the **Studies 4**, **5**, **7** and **8** corroborating previous data. Macroscopic lesions of the liver, lungs and trachea are presented in **Figure 18**. Microscopic lesions are showed in **Figure 19**.

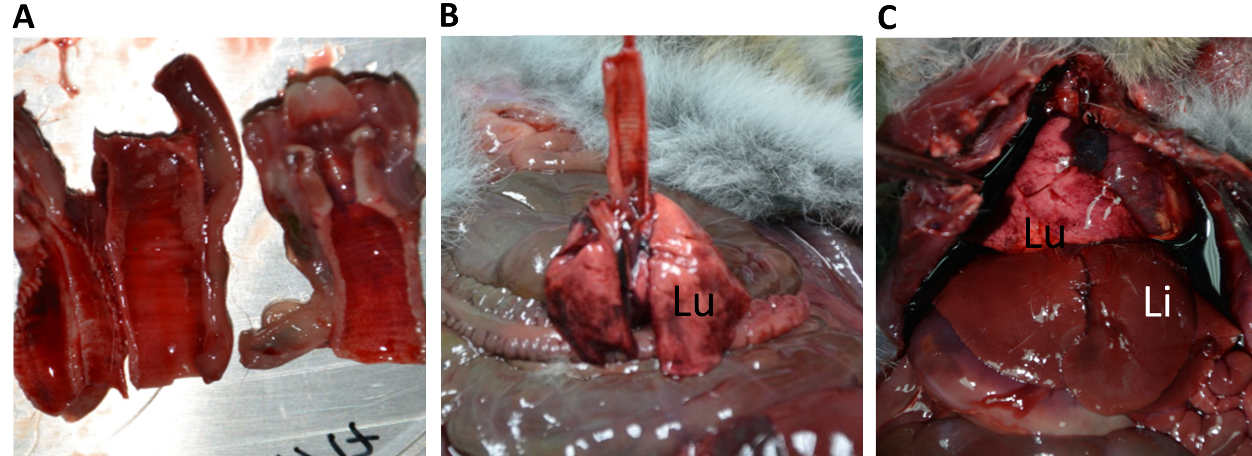


Figure 18. Necropsy of RHDV2-positive wild rabbits from Madeira Island (2017). **A**) Gross examination of longitudinal opened trachea showing a haemorrhagic mucosa; **B**) Lung congestion and haemorrhage of the trachea; **C**) Lung (**Lu**) congestion and marbled liver (**Li**). Photos kindly relinquished by Laboratório Regional de Veterinária e Segurança Alimentar (LRVSA), Madeira.

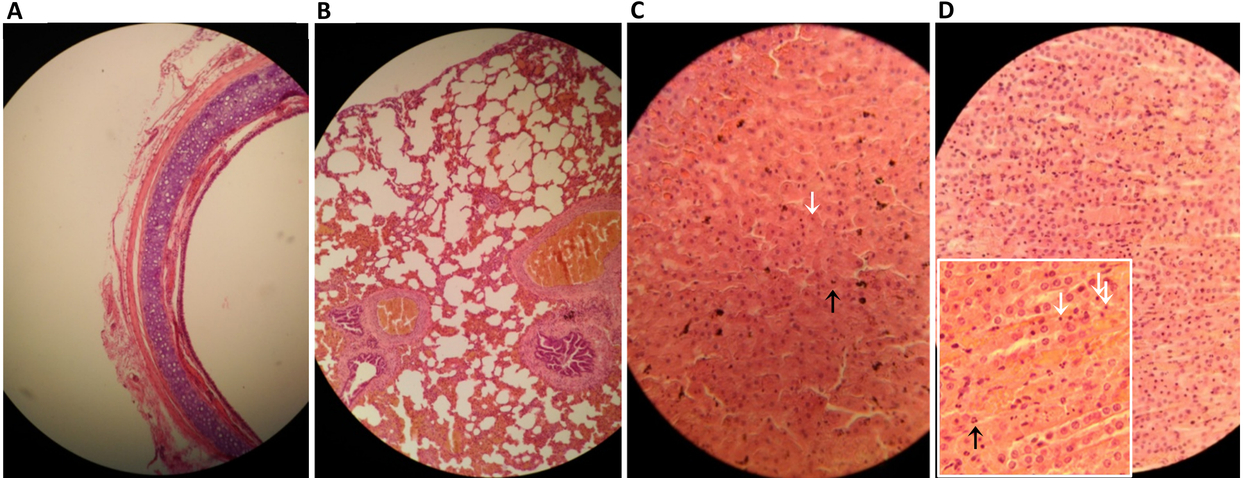


Figure 19. Histopathology of a wild rabbit collected during the Madeira Island 2017-RHDV2 outbreak (Haematoxylin & Eosin staining). **A)** Trachea exhibiting congestion of the mucosa (100x). **B)** Lung: congestion of the large vessels (100x). **C)** Liver, hepatocyte karyorrhexis (black arrow) and vacuolar degeneration (white arrow). Greenish brown pigment (hemosiderin) in the cytoplasm of hepatocytes and kupffer cells (200x). **D)** Kidney: picnosis (close-up, white arrows) and karyorrhexis (close- up, black arrow) of tubular cells (400x). Photos kindly relinquished by Laboratório Regional de Veterinária e Segurança Alimentar (LRVSA), Madeira.

2. Review on RHD prophylaxis

When RHDV emerged, vaccination was one of the first procedures recommended towards disease control but the implementation of sanitary measures was the most commonly adopted procedure. The control measures recommended by the OIE included the emergency slaughter of the complete rabbit populations of affected breeding farms, with repopulation after four weeks. "Sentinel rabbits" were introduced in small numbers in affected premises before complete repopulation (Argüello-Villares, 1991).

Still, control measures proved insufficient to guarantee protection against RHDV as disease cases still occurred in intensive establishments, reinforcing the need for vaccination (Argüello-Villares, 1991).

2.1. Active immunization

2.1.1. RHDV vaccines

Vaccination proved to be a valuable tool in disease control, reducing outbreaks and disease spread (Argüello-Villares, 1991). However, the lack of a suitable RHDV cell culture system has hindered large-scale production of the virus as a source of vaccine antigens (Pérez-Filgueira et al., 2007)(Gao et al., 2013). Some vaccines are still produced by the chemical inactivation of virus preparations obtained from liver tissues collected from experimentally infected rabbits (Argüello-Villares et al., 1991)(OIE Terrestrial Manual, 2016). This approach implicates handling large amounts of highly infectious material (Bárcena et al., 2000) while raising concerns about biological safety, contaminating residues, and animal welfare issues (Pérez- Filgueira et al., 2007)(Gao et al., 2013). Hence, research on RHDV vaccines based in biotechnology has been encouraged (OIE Terrestrial Manual 2016).

2.1.1.1. Inactivated tissue vaccines

The first report of RHDV inactivated vaccines date back to China where an inactivated vaccine for emergency vaccination was developed and a national vaccination programme was implemented (Huang, 1991). These vaccines were produced from infected liver after serial *in vivo* passages and inactivated in formalin were shown to have good immunogenicity with no adverse clinical reactions and induced immunity to persist until at least six months (Huang, 1991).

In alternative to liver, the spleen or kidney can be used to prepare the organ suspensions (Huang, 1991). Inactivation methods use formaldehyde, beta-propiolactone or other substances and the adjuvants such as incomplete mineral oil or aluminium hydroxide vary according to the protocol used by the different manufacturers (OIE Terrestrial Manual 2016).

The first injection should be given at 2–3 months (OIE Terrestrial Manual 2016). In farms with no history of disease and with negative serology for RHDV, only the breeding stock is to be vaccinated, independently of their age (OIE Terrestrial Manual 2016). Given the short life-cycle of fattening rabbits (approximately 80 days) and their natural resistance to the disease caused by RHDV/RHDVa up to the age of 6–8 weeks, vaccination of these rabbits is not necessary if no outbreaks of the disease in the farm or in the area have been recorded and good biosecurity measures are applied. However, this is not the case for RHDV2, which can affect younger animals, and this is explained ahead in point "2.1.2. RHDV2 vaccines".

21.1.1.1 Combined RHDV vaccines

The first report of combined RHDV vaccines occurred in China, referring to bivalent vaccines (RHD/pasteurellosis, RHD/clostridiosis, RHD/bordetellosis) and a trivalent vaccine (RHD/ pasteurellosis/clostridiosis) (Huang, 1991). In these combined vaccines the other components were shown to have no effect on RHDV immunogenicity and that the later did not influence the activity of the other components (Lin, 1987).

2.1.1.2. RHDV subunit recombinant vaccines

Parra and Prieto (1990) demonstrated that seroconversion to VP60 correlated with acquired immunity against RHDV and suggested that this viral polypeptide would be a good candidate for a subunit vaccine. Both active immunization with VP60 alone (Parra and Prieto, 1990)(Laurent et al., 1994) and passive immunization with anti-VP60 antibodies were reported to confer protection against RHDV challenge (Laurent et al., 1994). The production of recombinant VP60 would obviate the handling of infectious material for vaccine preparation (Laurent et al., 1994)(Sibilia et al., 1995). Aiming the production of safer vaccines, the *vp60* capsid gene have been successfully expressed in several heterologous systems, including bacteria (Boga et al., 1994), yeasts (Farnós et al., 2005), plants (Castañón et al., 1999)(Fernández-Fernández et al., 2001), poxvirus-based vectors (Bertagnoli et al., 1996a)(Bárcena et al., 2000) and insects cells using recombinant baculovirus (Laurent et al., 1994)(Nagesha et al., 1995). The RHDV VP60 expressed in most of these systems was shown to confer full protection against lethal challenge with RHDV in rabbits (Laurent et al., 1994)(Sibilia et al., 1995)(Boga et al., 1994)(Bertagnoli et al., 1996a)(Castañón et al., 1999)(Gao et al., 2013). Still, these vaccines field application has been restricted due to high production costs or low yield (Pérez-Filgueira et al., 2007)(Gao et al., 2013).

2.1.1.2.1 Recombinant RHDV-VP60 expressed in baculovirus/Sf9 cell expression system

The RHDV capsid protein was expressed in baculovirus/ Sf9 (*Spodoptera frugiperda* 9) cell expression system allowing high-level production of recombinant proteins (Laurent et al., 1994)(Sibilia et al., 1995). The recombinant proteins self-assembled into virus-like particles (VLPs), antigenically identical to RHD native virions that could be used for vaccination purposes (Laurent et al., 1994)(Sibilia et al., 1995). Intramuscular (i.m.) vaccination of rabbits with the VLPs (100 µg/ animal) conferred complete and effective protection in 15 days and was accompanied by a strong humoral response (Laurent et al., 1994). Anti-VP60 antibodies were detected as early as 5 days after

post-vaccination and the titres progressively increased over a 15-day period (Laurent et al., 1994). Nevertheless, the high amount of antigen (100 µg/ animal) required would render the vaccine very expensive from a practical perspective (Plana-Duran et al., 1996).

Plana-Duran et al. (1996) consider necessary to study the immunogenicity of the VLP particles by the oral route, making feasible to use these particles as an alternative to parenteral immunizations in field, for the vaccination of wild rabbits. They expressed the VP60 protein under the control of the polyhedrin and p10 promoter of baculovirus. As low as 3µg/ animal of RHDV-VLPs were able to induce significant titres of serum IgG antibodies conferring protection to the vaccinated rabbits, without the use of any adjuvant. For the stabilization of the VLPs, the use of binary ethylenimine was decisive by enabling the VLPs to pass the acidic and proteolytic barriers of the gut, without affecting the immunogenicity. Plana-Durant et al. (1996) found that the antibody titres obtained with oral vaccination, were, in some cases, superior to those obtained with a subcutaneous administration of the same dose. Each VP60-VLP contains 180 copies of the same protein, highly organized in a regular structure of 40 nm, which makes it an ideal candidate for the stimulation of different branches of the immune system (Plana-Duran et al., 1996).

The VP60 expression in the Sf9 cell expression system was optimized by Gao et al. (2013) according to the codon usage frequency of highly expressed genes in insect (Gao et al., 2013).

2.1.1.2.2. Escherichia coli recombinant RHDV subunit vaccine

Boga et al. (1994) expressed the RHDV capsid protein in *Escherichia coli* and demonstrated that the recombinant VP60 produced in the T7 RNA polymerase-based system was antigenically similar to the viral protein and able to protect the rabbits against RHDV (Boga et al., 1994).

Rabbits inoculated twice (one week interval) with 100 µg of purified VP60 survived challenge with lethal dose of RHDV administered intranasally (i.n.) 7 days after the last immunization (Boga et al., 1994). However, this vaccine was highly insoluble and of low immunogenicity (Plana-Duran et al., 1996).

2.1.1.2.3. RHDV recombinant vaccines using a myxoma virus vector

Bertagnoli et al. (1996a) reported the construction of two myxoma-RHDV recombinant viruses aiming the protection of rabbits against both diseases (Bertagnoli et al., 1996a). The recombinant myxoma viruses (MYXVs) were constructed based on the attenuated SG33 myxoma strain and expressed the RHDV capsid protein (VP60). The recombinant protein was antigenic and both

recombinant viruses induced high levels of RHDV and myxoma virus-specific antibodies in rabbits after immunization by the intradermal route (i.d.). Rabbits were protected against challenge with lethal dose of RHDV.

Bárcena et al. (2000) developed a recombinant vaccine based on a naturally attenuated MYXV field strain (6918) which expressed the RHDV VP60, capable of spreading through rabbit populations by horizontal transmission. This vaccine was developed aiming large-scale wild rabbit immunization against myxomatosis and RHD by the oral route. A linear epitope tag from the transmissible gastroenteritis virus (TGEV) nucleoprotein was included to monitor the spread and efficacy of the recombinant virus vaccine in the field and differentiate between naturally infected and immunized animals. This recombinant vaccine induced specific antibody responses against MYXV, RHDV, and the TGEV tag. Immunization of wild rabbits by the subcutaneous (s.c.) and oral routes conferred protection against virulent RHDV and MYXV challenges. Still, the recombinant viruses showed limited horizontal transmission, either by direct contact or in a flea-mediated process. The safety of this vaccine was evaluated by Torres et al. (2000) demonstrating that the recombinant virus maintained is attenuated phenotype after 10 passages *in vivo*. Their results showed that vaccine administration was safe even at a 100-fold overdose, with no undesirable effects upon administration to immunosuppressed or pregnant female rabbits.

More recently, Spibey et al. (2011) developed a recombinant MYXV-RHDV vaccine for the prevention of myxomatosis and RHD. This vaccine was shown to confer full and effective protection against challenged with pathogenic strains of RHDV and MYXV. Safety studies conducted in rabbits revealed no adverse clinical signs, even in young animals. MYXV-based vaccines may have safety concerns particularly in young rabbits, due to the immunosuppressive characteristic of the MYXV if not sufficiently attenuated (Spibey et al., 2012). In addition, the vaccine developed by Spibey et al. (2011) is unable to disseminate in the vaccinated rabbits beyond the local draining lymph node and the skin around the injection site and accounted for its good safety profile. This commercially vaccine is available in several countries for administration by the parenteral route (OIE Terrestrial Manual 2016).

2.1.1.2.4. Recombinant vaccinia-RHDV virus

A recombinant vaccinia-RHDV virus (Copenhagen strain) expressing the RHDV capsid protein VP60 was developed by Bertagnoli et al. (1996b). The recombinant virus (RecV-VP60) induced high level of RHDV specific antibodies in rabbits following immunization by the i.d. (0.1 ml) and oral routes

(10^9 pfu). The vaccine was shown to provide total protection against lethal RHDV, 15 days after immunization.

2.1.1.2.5. Canarypox-based RHDV recombinant vaccine

A canarypox-based recombinant virus expressing a native RHDV capsid protein (ALVAC-RHDV) was constructed by Fischer et al. (1997), the vCP309. The inoculation of high (10^7 pfu) or low (10^5 pfu) doses of vCP309 demonstrated to protect rabbits against a lethal RHDV challenge.

2.1.1.2.6. Insect larvae-derived recombinant RHDV subunit vaccine

Pérez-Filgueira et al. (2007) described the development of an inexpensive, safe and stable insect (*Trichoplusia ni*) larvae-derived recombinant subunit vaccine for RHDV. A baculovirus expressing a recombinant RHDV-VP60 (VP60r) was used to infect *T. ni* insect larvae. Yields of VP60r in larvae corresponded to ≈2 mg of recombinant protein per infected animal. The amount of VP60r obtained from 10 infected larvae corresponded to 10^9 cells of an infected Sf9 culture. According to Pérez-Filgueira et al. (2007), based on the production yields and i.m trial results, the amount of VP60r accumulated in a single larva would give ≈1000 i.m. vaccine doses. In addition, a simple extraction protocol can be used for recovering the VP60r from larvae that are suitable for immunization as crude preparations.

Rabbits immunized once by the i.m. route with an experimental oil vaccine formulated with complete Freun adjuvant containing 2 µg of VP60r were protected against challenge with lethal RHDV. The animals rapidly developed a systemic anti-RHDV response.

Oral immunization with encapsulated VP60r extracts was less reliable once, while some rabbits developed specific antibodies against RHDV, the humoral responses showed to be insufficient to provide protection against challenge with lethal RHDV.

2.1.1.2.7. RHDV recombinant subunit vaccine obtained from Pichia pastoris

Farnós et al. (2005) cloned the RHDV-VP60 from strains AST/89 and expressed it in in the yeast *Pichia pastoris*. The transformed yeast was grown at high cell density and an expression level of 1.5 g/ L of culture media was obtained. The protein, associated with the cell debris fraction, was purified and an N-glycosylated version was recovered with a purity of ≈70%. The recombinant RHDV-VP60 was antigenically similar to the native one. Immunization of rabbits s.c., generated a

virus-specific antibody response. In addition, the protein was able to protect rabbits immunized by the oral route against challenge with lethal RHDV injected i.m.

RHDV-VP60 associated with the disrupted pellet of *P. pastoris* was obtained insoluble under the SUC2 (*Saccharomyces. cerevisiae*) secretion signal and a laborious solubilisation process was needed. Farnós et al. (2009) proposed that the expression of a soluble variant of VP60, easier to purify, could represent a more appealing approach for vaccine production. They cloned the RHDV-VP60 under the transcriptional control of the *AOX*1 yeast promoter and the antigen obtained was intracellular and soluble at approximately 480 mg/L^{-1}. The antigenic profile was similar to that of native virions. Rabbits immunized twice 21 days apart with 50 µg of VP60 by the s.c. or three times with 0.5 mg of VP60 per dose by the oral route produced high titres of specific antibodies.

2.1.1.2.8. Adenovirus vectored vaccine against RHDV

Fernández et al. (2011) constructed two human type 5 derived replication-defective adenoviruses (Ad) encoding the RHDV-VP60, the AdVP60 vector. The recombinant protein was expressed as a multimer in mouse and rabbit cell lines at levels that ranged from 120 to 160 mg/L of culture. Immunization with human Ad5 derived replication-defective vectors elicited a potent and long-lasting immune response after parenteral or mucosal administration. Rabbits immunized by s.c. or mucosal (i.n.) routes with a single 10^9 GTU dose of the AdVP60 developed a strong IgG response, sufficient for providing complete protection against a lethal challenge with RHDV, and specific IgA antibodies in saliva.

2.1.1.2.9. Expression of the RHDV-VP60 antigen in transgenic plants

The VP60 structural protein have also been expressed in transgenic plants such as potato (Castañón et al., 1999)(Martín-Alonso et al., 2003)(Mikschofsky et al., 2011) or tobacco (*Arabidopsis thaliana*) (Gil et al., 2006). Edible plants for oral immunization may be a new mean for oral immunization of wild rabbits against RHDV (Martín-Alonso et al., 2003).

Castañón et al. (1999) produced the RHDV-VP60 in transgenic potato plants under the control of a cauliflower mosaic virus 35S promoter or a modified 35S promoter. Both types of promoters allowed the production of specific mRNAs and detectable levels of recombinant VP60, higher for the constructs carrying the modified 35S promoter. Adult rabbits immunized by the parenteral (s.c. or i.m.) route with 1 ml of leaf extracts from plants containing 12µg of recombinant VP60 were fully protected against challenge with RHDV. However, the resulting expression levels were inadequate

for the efficient use of tubers as vaccines, especially for oral administration requiring high antigen doses.

Martín-Alonso et al. (2003) also reported the production or recombinant RHDV-VP60 in transgenic tubers of potato plants and its use as an oral immunogen in rabbits. Animals were orally immunized with lyophilized transgenic tuber containing 100 µg or 500 µg of VP60 and three boosters were given 21 days apart. The antibody titters obtained in rabbits orally immunized with 100 µg of VP60 were undetectable and, overall, this vaccine was not protective against challenge.

Fernández-Fernández et al., (2001) constructed a new plum pox potyvirus (PPV)-based vector (PPV-NK) used for cloning different genes, engendering stable chimeras. The VP60 structural protein of RHDV was also successfully expressed by making use of the PPV–NK vector. The construct was able to infect *Nicotiana clevelandii* plants efficiently. Inoculation of rabbits with extracts from *N. clevelandii* expressing the VP60 induced a remarkable immune response against RHDV and the animals were protected against a lethal challenge.

2.1.2 RHDV2 vaccines

RHDV2 has distinct antigenic features in comparison to classical RHDV (Le Gall-Reculé et al., 2013)(Dalton et al., 2012)(Bárcena et al., 2015). Classical RHDV vaccines conferred only partial protection against this new virus not preventing infection (OIE Terrestrial Manual 2016). New specific RHDV2 inactivated vaccines have been developed and their use provisionally allowed in the European Union member states (Bárcena et al., 2015).

The first RHDV2 vaccine was developed in France (Filavac VHD variant, Laboratoire Filavie) in 2013 (Le Minoir et al., 2013) and reported to induce full and rapid protective immunity against RHDV2, showing no cross protection against RHDV (Le Minor et al., 2013). Spain also developed two specific vaccines against RHDV2, the Novarvilap vaccine (Ovejero Laboratories) intended for the vaccination of female breeders and passive immunization of kits, and Cunipravac RHD variant (Laboratorios Hipra, S.A). To our knowledge, there is no information publically available regarding the trials of both vaccines.

On 14 July 2016, the Committee for Medicinal Products for Veterinary Use (CVMP) recommended the granting of a marketing authorisation regarding the ERAVAC vaccine (Laboratorios Hipra, S.A.), intended for active immunisation of fattening rabbits against RHDV2 (http://www.ema.europa.eu). The efficacy of the ERAVAC vaccine was investigated in fattening rabbits under laboratory conditions using an adequate challenge model, with a challenge strain different form the one

included in the vaccine. The vaccine was shown to provide protection only against RHDV2, with no cross protection against classical RHDV. Onset of immunity was established 7 days after vaccination although the duration of immunity has not been fully established. The main reported adverse reaction was a slight transient increase in rectal temperature, which resolved spontaneously (http://www.ema.europa.eu).

The protocols recommended for the administration of the RHDV2 specific inactivated vaccines are similar to those for classical RHDV. However, as RHDV2 can induce disease in younger animals (Dalton et al., 2012), following an outbreak it is strongly recommended to vaccinate meat animals at the age of 30–40 days, even if strict hygiene and sanitary measures are adopted, including cleaning and disinfection, safe disposal of carcasses and an interval before restocking, for incidence of re-infection is very high . Vaccination should only be interrupted after several production cycles. In addition, to verify the persistence of infective RHD inside the farm, sentinel rabbits should not be vaccinated (OIE Terrestrial Manual 2016).

In 2015, a recombinant baculovirus expressing the RHDV2 VP60 was also assembled providing insights regarding the marked RHDV2 antigenic differences and opening the way to the development of new recombinant RHDV2 subunit vaccines (Bárcena et al., 2015). Similar to what was observed for RHDV, these recombinant baculovirus could also be useful tools for RHDV2 circulation monitoring, development of control measures and research (Bárcena et al., 2000).

2.1.3. Vaccine as post-exposure treatment at the population level

Rabbits with light or none clinical signs could recover after emergency inoculation with inactivated tissue vaccine (Huang, 1991). Huang et al. (1986) demonstrated that the majority of experimentally RHDV-infected rabbits were able to resist RHD after three to fourfold doses of RHDV vaccine, although their recovery was slower.

Given that immunity starts earlier (7–10 days after immunization), vaccination can be considered a quite effective post-exposure treatment at the population level. In particular situations, it may be included in the emergency strategies applied when RHD occurs on farms with separate sheds and good biosecurity measures (OIE Terrestrial Manual, 2016). We were given the opportunity to evaluate the effectiveness of vaccination for RHDV2 as a therapeutic tool in **Study 5**.

2.2. Passive immunization

In China, hyperimmune antiserum was used for emergency inoculation aiming the quick establishment of short-term immunity and treatment of infected animals by neutralising the virus (Huang, 1991). However, it was only effective in infected rabbits with light or no clinical signs (Huang, 1991). Hyperimmune sera produce a rapid but short-lived protection against RHDV infection, limiting the spread of the disease and reducing economic losses. Similarly to what happens for vaccines, it is necessary to use sera homologous to the causative RHDV strain (OIE Terrestrial Manual 2016).

Study 5

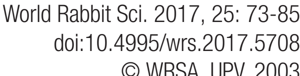
Is vaccination an important tool for the control of RHDV2 ongoing infections?

Progression of rabbit haemorrhagic disease virus 2 upon vaccination in an industrial rabbitry: a laboratorial approach

Carina L. Carvalho[1], Elsa L. Duarte[1,2], José M. Monteiro[3], Carla Afonso[4], João Pacheco[4], Paulo Carvalho[5], Paula Mendonça[5], Ana Botelho[5], Teresa Albuquerque[5], Patrícia Themudo[5], Miguel Fevereiro[5], Ana M. Henriques[5], Sílvia S. Barros[5], Margarida D. Duarte[5]

[1]Instituto de Ciências Agrárias e Ambientais Mediterrânicas (ICAAM), Universidade de Évora, Évora, Portugal; [2]Departamento de Medicina Veterinária, Universidade de Évora, Évora, Portugal; [3]Sumicor Portugal, Lda., Vila do Conde, Portugal; [4]Segalab, Laboratório de Sanidade Animal e Segurança Alimentar, Argivai, Portugal; [5]Instituto Nacional de Investigação Agrária e Veterinária (INIAV), Lisboa, Portugal;

World rabbit Science Journal DOI: https://doi.org/10.4995/wrs.2017.5708


World Rabbit Sci. 2017, 25: 73-85
doi:10.4995/wrs.2017.5708
© WRSA, UPV, 2003

PROGRESSION OF RABBIT HAEMORRHAGIC DISEASE VIRUS 2 UPON VACCINATION IN AN INDUSTRIAL RABBITRY: A LABORATORIAL APPROACH

CARVALHO C.L.[*], LECLERC DUARTE E.[*†], MONTEIRO J.M.[‡], AFONSO C.[§], PACHECO J.[§], CARVALHO P.[#], MENDONÇA P.[#], BOTELHO A.[¶], ALBUQUERQUE T.[¶], THEMUDO P.[¶], FEVEREIRO M.[||], HENRIQUES A.M.[||], SANTOS BARROS S.[||], DIAS DUARTE M.[||]

[*]ICAAM-Instituto de Ciências Agrárias e Ambientais Mediterrânicas, Instituto de Investigação e Formação Avançada, Universidade de Évora, Núcleo da Mitra, 7000 Évora, Portugal.
[†]Departamento de Medicina Veterinária, Universidade de Évora, Núcleo da Mitra, 7000 Évora, Portugal.
[‡]Sumicor Portugal, Lda., Rua nº3, Lote 11, Zona Industrial da Varziela, Apart. 74, 4481-911 Vila do Conde, Portugal.
[§]Segalab, Laboratório de Sanidade Animal e Segurança Alimentar, Lugar de Cassapos, 4490-258 Argivai, Portugal.
[#]Instituto Nacional de Investigação Agrária e Veterinária (INIAV), Laboratorio de Patologia, Av. da Républica, Quinta do Marquês, 2780-157 Oeiras, Portugal.
[¶]Instituto Nacional de Investigação Agrária e Veterinária (INIAV), Laboratorio de Bacteriologia, Av. da Républica, Quinta do Marquês, 2780-157 Oeiras, Portugal.
[||]Instituto Nacional de Investigação Agrária e Veterinária (INIAV), Laboratorio de Virologia, Av. da Républica, Quinta do Marquês, 2780-157 Oeiras, Portugal.

Abstract: Rabbit haemorrhagic disease virus 2 (RHDV2) emerged recently in several European countries, leading to extensive economic losses in the industry. In response to this new infection, specific inactivated vaccines were developed in Europe and full and rapid setup of protective immunity induced by vaccination was reported. However, data on the efficacy of these vaccines in an ongoing-infection scenario is unavailable. In this study we investigated an infected RHDV2 indoor industrial meat rabbitry, where fatalities continued to occur after the implementation of the RHDV2 vaccination, introduced to control the disease. The aim of this study was to understand if these mortalities were RHDV2-related, to discover if the dead animals showed any common features such as age or time distance from vaccination, and to identify the source of the outbreak. Anatomo-pathological analysis of vaccinated animals with the virus showed lesions compatible with systemic haemorrhagic disease and RHDV2-RNA was detected in 85.7% of the animals tested. Sequencing of the *vp60* gene amplified from liver samples led to the recognition of RHDV2 field strains demonstrating that after the implementation of vaccination, RHDV2 continued to circulate in the premises and to cause sporadic deaths. A nearby, semi-intensive, RHDV2 infected farm belonging to the same owner was identified as the most probable source of the virus. The main risk factors for virus introduction in these two industries were identified. Despite the virus being able to infect a few of the vaccinated rabbits, the significant decrease in mortality rate observed in vaccinated adult rabbits clearly reflects the efficacy of the vaccination. Nonetheless, the time taken to control the infection also highlights the importance of RHDV2 vaccination prior to the first contact with the virus, highly recommendable in endemic areas, to mitigate the infection's impact on the industry.

Key Words: rabbit, RHDV2, rabbit haemorrhagic disease, vaccines.

INTRODUCTION

Both rabbit haemorrhagic disease virus 2 (RHDV2) and rabbit haemorrhagic disease virus (RHDV) are classified within the *Lagovirus* genus along with the genetically related European brown hare syndrome virus (EBHSV)(Capucci *et al.,* 1991; Le Gall-Reculé *et al.,* 2013). RHDV2 was first reported in 2010 (Le Gall-Reculé *et al.,* 2011), whereas classic

Correspondence: M. Dias Duarte, margarida.duarte@iniav.pt. Received *May 2016* - Accepted *November 2016.*
doi:10.4995/wrs.2017.5708

RHDV strains have circulated worldwide since 1984 (Nowotny *et al.,* 1997; Capucci *et al.,* 1998; Le Gall *et al.,* 1998; Le Gall-Reculé *et al.,* 2003).

Although rabbit haemorrhagic disease (RHD) was first described in China during the 1980s in Angora rabbits that had been imported from Germany, the virus is thought to have originated in Europe (Liu *et al.,* 1984). It causes a lethal and contagious disease which presents with liver necrosis, splenomegaly and haemorrhagic lesions in the liver and in the lungs (Capucci *et al.,* 1991; Marcato *et al.,* 1991). Typically, diseased animals present with fever (>40°C), and death occurs rapidly within 12 to 36 h after exposure (Capucci *et al.,* 1991; Marcato *et al.,* 1991; McIntosh *et al.,* 2007). Common clinical signs include a blood-tinged foamy nasal discharge, severe respiratory distress and/or convulsions, usually preceding death (Marcato *et al.,* 1991; McIntosh *et al.,* 2007). Mortality rates are high, ranging from 80% to 100% (Marcato *et al.,* 1991; Ohlinger *et al.,* 1993).

RHDV2, also known as RHDVb, is closely related to RHDV but also highly genetically distinctive and therefore represents a new genotype (Le Gall-Reculé *et al.,* 2011, 2013). After its emergence in France (Le Gall-Reculé *et al.,* 2011), it quickly spread to other European countries (revised in Duarte *et al.,* 2015a), replacing the classic strains previously circulating in France (Le Gall-Reculé *et al.,* 2013), the Iberian Peninsula (Bárcena *et al.,* 2015; Calvete *et al.,* 2014; Dalton *et al.,* 2014; Lopes *et al.,* 2015) and the Azores (Duarte *et al.,* 2015a). RHDV2 was also reported in an isolated case in Australia (Hall *et al.,* 2015). In addition to the European rabbit, RHDV2 is able to infect a few hare species (Puggioni *et al.,* 2013; Camarda *et al.,* 2014).

RHDV2 is less virulent than RHDV, and therefore associated with lower mortality rates (Le Gall-Reculé *et al.,* 2013). The disease developed also differs from that induced by RHDV in its clinical presentation, given that chronic and sub-acute forms are more frequent in RHDV2 infections (Le Gall-Reculé *et al.,* 2013) whereas in RHDV infections only a small percentage of animals (5 to 10%) develop a sub-acute or chronic illness presenting with jaundice, malaise, weight-loss and death within 1 to 2 wk after the onset of symptoms (McIntosh *et al.,* 2007).

Nestlings as young as 11 d old are susceptible to RHDV2 and develop the disease (Dalton *et al.,* 2012), unlike in the case of RHDV, to which kits up to 4 wk of age are naturally resistant (Liu *et al.,* 1984). Regardless of age-independent susceptibility, the lack of cross protection induced by previous contact with RHDV strains contributed to the rapid spread of RHDV2 in Europe (Le Gall-Reculé *et al.,* 2013), resulting in high mortality rates among wild populations soon after its emergence.

In the industry, RHDV2 was reported for the first time in France (Le Gall-Reculé *et al.,* 2011), and soon after in Great Britain (Baily *et al.,* 2014; Westcott *et al.,* 2014) and the Iberian Peninsula (Dalton *et al.,* 2012; Duarte *et al.*, unpublished results), resulting in severe losses in this sector. RHDV-vaccinated rabbits are totally protected against RHDV infection but only partially to RHDV2. Hence, in farms with no RHDV vaccination in place the mortality rates induced by RHDV2 rose to 80%, while in vaccinated animals the rate observed was considerably lower (25%) (Le Gall-Reculé *et al.,* 2013). Still, the limited cross protection against RHDV2 conferred by inactivated or recombinant RHDV vaccines (Torres *et al.,* 2000; Le Gall-Reculé *et al.*, 2011, 2013; Dalton *et al.,* 2014) led to the development of specific RHDV2 inactivated vaccines in Europe (Filavac VHD Variant, Filavie Laboratories, Cunipravac RHD variant, Hipra; Novarvilap, Ovejero). These vaccines were provisionally allowed in the European Union member states, as their use requires special licenses from the Local Veterinarian Authorities, and vaccination against RHDV2 has become common practice in the industry.

The full and rapid setup of RHDV2 protective immunity induced by Filavac VHD Variant vaccine, established within one week, was reported in four- and 10-wk old rabbits (Minor *et al.,* 2013). No cross protection against the classic virus was observed (Le Minor *et al.,* 2013). However, there is no data available on the use of vaccination in an ongoing-RHDV2 infection scenario.

In this study, we investigated a series of fatalities that occurred in an indoor industrial meat rabbitry after the implementation of the RHDV2 vaccination. To clarify the cause of the fatalities, we gathered and integrated clinical, epidemiologic, anatomo-histopathologic, virologic and bacteriologic data. After the identification and molecular characterisation of RHDV2 as the etiological agent, the research focused on detection of the probable infection source, transmission routes and the identification of possible reasons for infection recurrence after vaccination. Our data allow preliminary conclusions on the efficacy of vaccination as a therapeutic measure.

MATERIALS AND METHODS

Sample origin and epidemiological inquiry

A total of twenty-one dead rabbits were analysed. Nine rabbits had not been vaccinated and 12 were vaccinated once or twice against RHDV2. Fourteen of these 21 animals originated from the indoor, semi-intensive, meat rabbitry investigated in this study, located in the north of Portugal, hereby referred to as *cv-Farm* due to its controlled ventilation system. The other 7 samples originated from a second industrial farm, located 300 m away, referred to as *nv-Farm,* given its natural ventilation. The casualties occurred between January 2015 and August 2015 (Table 1). The cadavers or organs were received at a private laboratory, Segalab, S.A., and then sent to the Instituto Nacional de Investigação Agrária e Veterinária (INIAV).

An inquiry was carried out to gather information at the cv-Farm facilities, its operating system, production rates, reproductive strategies, prophylactic measures, and, regarding the 21 specimens' age, immunisation profile and vaccination date, time of death, and necropsy data (if performed at the rabbitry). Information on the overall mortality among adults and kits was also collected. Cases were numbered according to their chronological occurrence.

Anatomo-histopathological examination

Necropsies were performed by the veterinarian assistant at the rabbitry or by the pathologists at the Pathology Laboratory, INIAV.

For anatomo-pathological examinations, liver and lung samples were fixed in 10% buffered formalin and embedded in paraffin using standard procedures. Five micrometre-thick sections were stained with haematoxylin and eosin (H&E) and examined using light microscopy (Cook, 1997).

Bacteriological analysis

Liver and lung samples from the 21 animals were analysed using standard bacteriological methods, and the presence of *Pasteurella* sp*.,* which is to be considered in the differential diagnosis of RHD according to the OIE (World Organisation for Animal Health (OIE) Technical disease cards, 2015), was investigated. Lung and liver sample macerates were inoculated in MacConkey agar (Oxoid) and Colombia agar (Oxoid), supplemented with 5% of defibrinated sheep blood (Biomerieux) and incubated at 37°C for 24-48 h. Identification of isolates was performed using the commercial API® test strips API 20 NE and API ID32 E (BioMérieux). To infer the sanitary status of the animals, which is a public health concern as they are used for human consumption, the presence of verocytoxin (VT) producing *Escherichia coli* strains (VTEC) was investigated by multiplex PCR (Paton and Paton, 1998). *E. coli* strains ED647 (*E. coli* 0157*, vt1, vt2, eae*) and ED378 (*018ab*, *vt2f*), provided by the European Reference Laboratory for VTEC, Instituto Superiore di Sanitá Italy, were used as positive controls and *E. coli* strain JM109 as a negative control.

Virological examination

Tissue samples comprising liver and lungs were homogenised with phosphate buffered saline (PBS) and clarified at 3000 *g* for 5 min. DNA and RNA were extracted from 200 µL of the clarified supernatant, corresponding to approximately 50 mg of tissue, in a BioSprint 96 nucleic acid extractor (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

With regard to vaccines, RNA from vaccine Cunipravac RHD variant (Hipra, Spain) was extracted from the aqueous phase of a centrifuged sample (10000 *g* for 10 min), using the RNeasy blood and tissue kit (Qiagen, Hilden, Germany) according to the recommendations. RNA from vaccine Novarvilap (Ovejero, Spain) was extracted from 200 µL of a 10× diluted sample (v/v in bidistilled H_20), in a BioSprint 96 nucleic acid extractor (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Samples were tested for RHDV2 by RT-qPCR (Duarte *et al.,* 2015b). Screening for RHDV was performed by sequencing analysis of the amplicons obtained by conventional PCR with primers RC-9 and RC-10 (Tham *et al.,* 1999). Conventional RT-PCR and RT-qPCR were performed using the One Step RT-PCR kit (Qiagen, Hilden, Germany).

Table 1: Information on the cases investigated; age at the time of death, vaccination history, virological laboratorial results and accession numbers of vp60 gene sequences.

					Vaccinations					Virological examination		
Case No.	Date of death	Age group	GenBank accession #*vp60* sequence	Farm of origin	MYXV Mixohipra	RHDV Arvilap	RHDV2 vaccine Novarvilap (1) Cunipravac (2)	Vaccination date for RHDV2	Time gap (d) between last vaccination and death	RHDV2 (Cq)	RHDV	MYXV
0	22 Oct14	Adult	KU665601[c]	nv-Farm	Y	Y	-	Not vaccinated	-	12.58	N	N
1	28 Oct14	Adult	KU665600[c]	cv-Farm	Y	Y	-	Not vaccinated	-	11.83	N	N
2	06 Jan15	Adult	ND	cv-Farm	Y	Y	1	30 Oct14	68	31.71	N	N
3	06 Jan15	Adult	KU665598[c]	cv-Farm	Y	Y	1	30 Oct14	68	9.53	N	N
4	03 Feb15	Adult	-	cv-Farm	Y	Y	1	30 Oct14	96	no Cq	N	N
5	28 Feb15	Adult	ND	cv-Farm	Y	Y	1	30 Oct14	121	33.09	N	N
6	28 Feb15	Adult	ND	cv-Farm	Y	Y	1	30 Oct14	121	32.17	N	N
7	17 Mar15	Adult*	KU665597[c]	cv-Farm	Y	Y	1,2	30 Oct14,02 Mar15	138,15	15.27	N	N
8	17 Mar15	Adult[+]	-	cv-Farm	Y	Y	1,2	30 Oct14,02 Mar15	138,15	29.08	N	N
9	17 Mar15	Young (35d)	ND	cv-Farm	Not vaccinated	Not vaccinated	2	02 Mar15	15	16.85	N	N
10	17 Mar15	Young (35d)	KU665599[p]	cv-Farm	Not vaccinated	Not vaccinated	2	02 Mar15	15	11.82	N	N
11	17 Mar15	Young (35d)	ND	cv-Farm	Not vaccinated	Not vaccinated	2	02 Mar15	15	12.74	N	N
12	29 Mar15	Adult	ND	cv-Farm	Y	Y	-	Not vaccinated	-	11.56	N	N
13	29 Mar15	Adult	ND	cv-Farm	Y	Y	1	02 Mar15	27	36.14	N	N
14	20 Apr15	Adult	-	cv-Farm	Y	Y	2	02 Mar15	48	no Cq	N	N
15	22 Apr15	Young (>35d)	KU665594[c]	nv-Farm	Not vaccinated	Not vaccinated	-	Not vaccinated	-	16.22	N	N
16	22 Apr15	Young (>35d)	KU665596[c]	nv-Farm	Not vaccinated	Not vaccinated	-	Not vaccinated	-	17.29	N	N
17	22 Apr15	Young (>35d)	KU665595[c]	nv-Farm	Not vaccinated	Not vaccinated	-	Not vaccinated	-	22.46	N	N
18	24 Jul15	Young (59d)	ND	nv-Farm	Not vaccinated	Not vaccinated	-	Not vaccinated	-	21.60	N	N
19	24 Jul15	Young (59d)	ND	nv-Farm	Not vaccinated	Not vaccinated	-	Not vaccinated	-	20.10	N	N
20	18 Aug15	Young (>35d)	-	nv-Farm	Not vaccinated	Not vaccinated	-	Not vaccinated	-	no Cq	N	N

ND: Not determined; *Multiparous doe, that had given birth 11 d prior; +: Multiparous doe, in the initial stage of pregnancy; Y: Yes; N: Negative; cv-Farm: controlled ventilated Farm; nv Farm: natural ventilated Farm; c: Complete; p: partial

The presence of myxoma virus was examined by qPCR (Duarte *et al.,* 2014), using the FastStart TaqMan Probe Master Kit (Roche, Roche Diagnostics GmbH, Manheim, Germany).

Cq (quantification cycle) values are inversely related to the concentration of nucleic acid in the sample (revised by (Gullett and Nolte, 2015)). For the real time PCR systems described, undetectable Cq or Cq values >40 were considered negative.

Nucleotide sequencing analysis

Amplification of the *vp60* sequences of RHDV2 strains was accomplished with 2 pairs of primers, 27F (5'-CCATGCCAGACTTGCGTCCC-3') and 986R (5'-AACCATCTGGAGCAATTTGGG-3'), 717F (5'-CGCAGATCTCCTCACAACCC-3') (Duarte *et al.,* 2015a), and RC10R (Tham *et al.,* 1999) enabling us to obtain 2 overlapping fragments. The One Step (Qiagen, Hilden, Germany) kit was used, following the manufacturers' recommendations. Sequencing was accomplished using the BigDye™ Terminator cycle sequencing kit (Applied Biosystems, Foster City, CA, USA).

The *vp60* nucleotide sequences of 7 complete and 1 partial RHDV2 strains (GenBank accession numbers KU665594 to KU665601) were determined in an automated 3130 Genetic Analyzer system (Applied Biosystems, Foster City, CA, USA).

Nucleotide diversity (π), and its corresponding variances (Nei, 1987) were estimated independently for each sub-population (nv-Farm and cv-Farm strains) as well as for both sub-populations combined, resorting to DNASP software (Version 5.10.01) (Rozas *et al.,* 2003). Standard errors (SE) of each measure were based on 1000 bootstrap replicates. DnaSP was also used to calculate the minimum number of recombination events (Rm) in the sample (Hudson and Kaplan, 1985).

RESULTS

Insights from the epidemiologic inquiry

The inquiry revealed that the animals originated from a high-standard rabbitry (cv-Farm) holding 800 does which produce around 6000 animals per productive cycle. Does, weighing between 3.5 and 4.5 kg, give birth every 42 d as artificial insemination is practiced 11 d postpartum, producing an average of 22 kg of meat per inseminated doe. The cv-Farm comprises separate areas for artificial breeding and fattening, and works in a closed, all-in-all-out system, with controlled temperature (22 to 26°C), humidity, artificial light and ventilation. Kits are weaned at 32 d of age, at which point the mothers are moved to a contiguous area where the next productive cycle begins. Fattened rabbits are collected for slaughtering at 70 d of age. Facilities are disinfected by a specialised company and kept empty for a week before the following breeding cycle. We also found out that a second semi-intensive farm (nv-Farm), belonging to the same owner, is located 300 metres away from the cv-Farm. Both farms are sited on agricultural land. No movement of animals was carried out between farms, but sharing of workers was identified.

Epidemiological data linking the farms is schematised in Figure 1.

Regarding the prophylactic measures in practice prior to RHDV2 introduction, it was disclosed that disease control was carried out by the veterinary assistant according to an established programme. RHDV vaccination (Arvilap) was carried out twice a year (spring and autumn), and Myxoma virus vaccination (MYXV) (Mixohipra H, Hipra) every 4.5 mo. Control of internal parasites is achieved by administering albendazole, levamisole, or fenbendazole, alternately, every other productive cycle. Insecticides are regularly used for mosquito control. Other preventive sanitary prophylactic measures include rodent and plague control by a certified company, which periodically monitors the installed devices (such as bait boxes with raticide). Ventilators are sited far from the communication doors and thus from any contact with the slaughter and food vehicles as well as the entries through which the animals enter and exit. In addition, cadavers are collected 2 km away from the farm by a specialist company, so that this vehicle does not approach the farm's surrounding area. There are no specific nets to avoid contacts with wild rabbits. All the farm workers use specific equipment and visitors are not allowed.

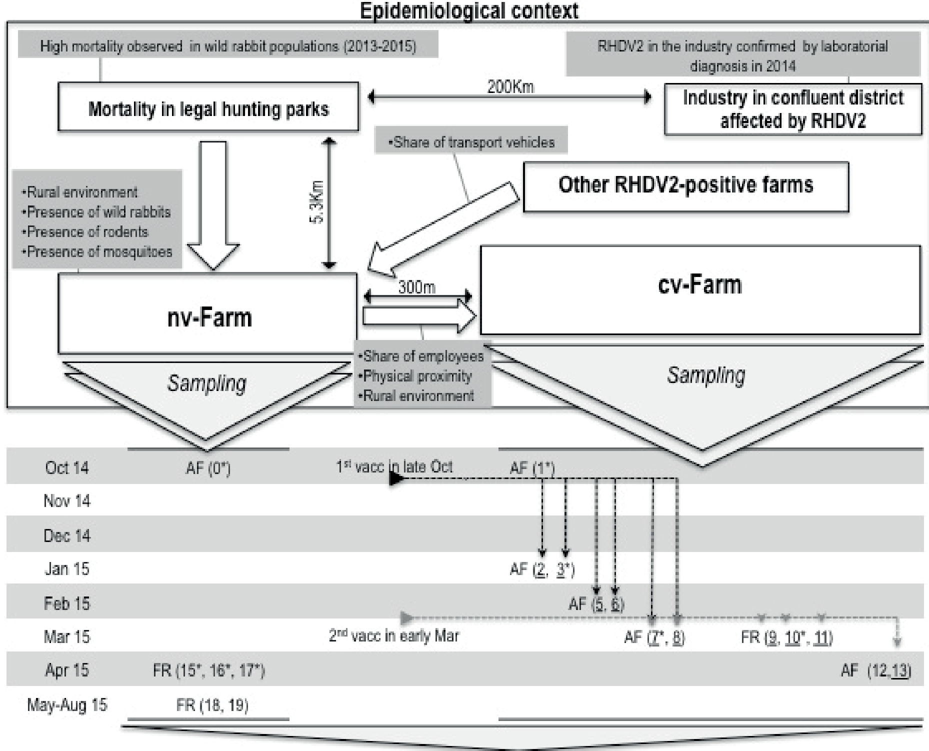


Figure 1: Top, schematic representation of the epidemiological context of the farm. Bottom, information on the RHDV2-positive sampling with regards to farm of origin, date of collection and vaccination. Black arrows represent geographical distances (not in scale). White arrows represent the possible origin and dissemination paths. Risk factors are shown within grey boxes. Numbers in brackets refer to case numbers, which position in relation to the calendar (on the left) refers to time of death. The case numbers corresponding to vaccinated rabbits are underscored. AF, adult females; FR, fattening rabbits; nv-Farm, natural ventilated farm; cv-Farm, controlled ventilated farm. Vaccines are represented by triangles (black, Novarvilap; grey, Cunipravac RHD variant). *Strains with *vp60* gene sequenced.

The events started in late October 2014, when a series of deaths were registered in the cv-Farm, with mortalities of 80% in juveniles and 30-40% in does (Case 1, adult female), respectively. The enquiry revealed that a week earlier, several animals of all ages had begun to die in the nv-Farm (Case 0, adult female). RHDV2 presence was confirmed in both farms by laboratory diagnosis and, RHDV2 vaccination of the adult females was implemented with Novarvilap (Ovejero Laboratories).

Two months later, in January 2015, 2 pregnant females from the cv-Farm, which had been vaccinated 68 d earlier, died suddenly without any clinical signs of disease (cases 2 and 3, Table 1). Macroscopic examination of these rabbits revealed good body condition, but haemorrhages in the lungs, icteric liver and splenomegaly, raising the suspicion of RHD. RHDV2 was confirmed by laboratorial diagnosis. A second RHDV2 outbreak was observed in the cv-farm in late February to early March. Newly weaned 27 d-old kits and 35 and 60 d-old rabbits were affected, most of which were offspring of primiparous females vaccinated once, 121 d prior. Adults also fell victim and lesions compatible with haemorrhagic disease were observed in a few females vaccinated 121 d earlier (cases 5 and 6, Table 1).

Limitations from the supplier led to the introduction of a second RHDV2 vaccine (Cunipravac RHD variant, Hipra, Spain) in both farms at the beginning of March and the enquiry revealed that a few weaned kits were also vaccinated once with this vaccine.

The last mortalities in the cv-Farm occurred in mid-late March, affecting vaccinated females (cases 7, 8, 13, Table 1), including one multiparous that had given birth recently (case 7) and another pregnant multiparous (case 8). Females 7 and 8 had been vaccinated twice, with the Norvavilap and Cunipravac RHD variant, 138 and 15 d before, respectively. Female 13 was vaccinated once with the Cunipravac RHD variant, 27 d before. Thirty-five-day old kits, vaccinated once with the Cunipravac RHD variant (cases 9, 10, 11, Table 1), also fell victim.

In the nv-Farm, mortalities due to RHDV2 continued to occur until late July 2015, affecting non-vaccinated fattening kits aged 35 to 70 d (cases 15-19, Table 1). No more casualties were observed thereafter.

Microbiology

RHDV2 was detected in 18 of the 21 rabbits analysed (Cases 0-3, 5-13, 15-19, Table 1, and Figure 2), by RT-qPCR (Duarte *et al.,* 2015b). Viral loads were variable, as Cq values ranged from 9.53 to 36.14, with high viral charges (Cq<25) found in the majority of the samples tested (n=13/18, 72.22%). Despite the limited sampling, a positive association was observed between viral loads and lesion severity in kits (Figure 2).

Only 3 out of the 21 animals analysed were negative for RHDV2 (cases 4, 14 and 20, Table 1). Data of the 18 RHDV2-positive cases, regarding sample origin, collection and vaccination date, are represented in Figure 1 (bottom).

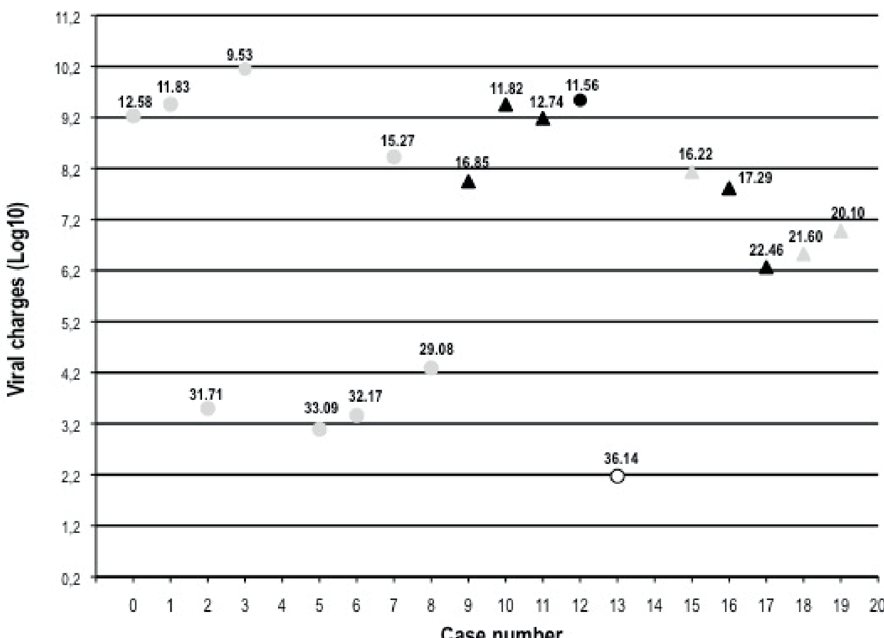


Figure 2: Viral charges found in RHDV2-positive samples. Circles represent adults and triangles the young. Colour corresponds to samples where microscopic lesions were observed (black), not determined (grey) or absent (white). Viral charges were inferred from the respective Cq values according to the regression equation published by Duarte *et al.*, 2015b. Cq values are shown above the symbols. Cases 4, 14 and 20 (RHDV2-negative) are not represented.

Pasteurella multocida was isolated from the lungs of one RHDV2-negative multiparous doe (case 4). Neither *Salmonella*, *Yersinia*, *Staphylococcus* nor *Listeria* were detected. *E. coli* was isolated from the tissues of 3 adult females (cases 5, 6 and 7) and 3 kits (cases 9, 10 and 11), all RHDV2-positive rabbits, but no associated virulence genes (*vtx1, vtx2, eae and vtx2f)* were found.

Anatomo-histopathology

No signs of disease were registered prior to death in any of the 21 rabbits. Bloody discharges from the nose, observed in 5 animals (23.81%, case 4 [RHDV2-negative], and cases 7, 9, 10 and 11, all RHDV2-positive), were the only outward manifestation of an ongoing pathology.

Macroscopically, in the RHDV2 positive animals, an icteric liver and hepatomegaly were the most common lesions (90 to 100%) followed by lung petechiae (45 to 50%) and moderate splenomegaly (30%). Hepatic discoloration was also observed (cases 9, 10 and 11, all referring to RHDV2 positive kits).

At microscopic level, the great majority of the lesions in the RHDV2-positive rabbits matched the typical RHD lesions described above (Ohlinger *et al.,* 1993). Those included microfoci of hepatic necrosis, detected in the liver parenchyma of 6 animals (cases 9-12, 16 and 17, all RHDV2-positive; cases 9-11 and 17 vaccinated, and cases 12 and 16 not vaccinated). Hepatocyte hyalinisation was found in 5 of these samples (cases 9-11, 16 and 17). Lung histopathology was performed in 2 specimens showing severe congestion and disseminated intravascular coagulation in the small capillaries (cases 9 and 10).

In the adult female case 4, lesions consisted of purulent pneumonia with extensive parenchymal infiltration by inflammatory cells, mainly neutrophils, and occasional bacteria clumps associated with necrotic foci.

Despite some degree of autolysis, no lesions were recognised in the liver or lungs of the adult rabbit case 13, where a RHDV2 high Cq value (36.14) was obtained.

Insights from the molecular analysis

Similarity among field strains was above 99.43%, with 2 of the complete sequences being identical (KU665597 and KU665598, Table 2) and 2 differing only in 1 nucleotide (KU665595 and KU665596, Table 2). The average number of nucleotide differences (k) and the nucleotide diversity (π) found among all *vp60* sequences showed intermediate

Table 2: Nucleotide similarity percentage among the *vp60* nucleotide sequences of two vaccines and eight field RHDV2 strains.

Strains	Case 0	Case 1	Case 3	Case 7	Case 10*	Case 15	Case 16	Case 17	Vaccine A	Vaccine B
Case 0#	-	100	99.60	99.83	99.90*	99.89	99.83	99.83	98.74	97.82
Case 1†		-	99.60	99.83	99.90*	99.89	99.83	99.83	98.74	97.82
Case 3†			-	99.43	99.18*	99.48	99.54	99.54	98.91	97.76
Case 7†				-	99.90*	99.83	99.77	99.77	98.56	97.64
Case 10†*					-	-*	99.80*	99.80*	98.17*	97.66*
Case 15#						-	99.83	99.83	98.62	97.70
Case 16#							-	99.94	98.68	97.64
Case 17#								-	98.68	97.64
Vaccine A									-	97.82
Vaccine B										-

Strains are referred by the case number.

*Similarities based on a partial *vp60* sequence (981bp long). Vaccines' identification is restrained for privacy.

#Lines highlight samples from the semi-intensive farm.

†Lines correspond to samples originated in the industrial farm.

GenBank accession numbers: Case 0 (KU665601), Case 1 (KU665600), Case 3 (KU665598), Case 7 (KU665597), Case 10 (KU665599), Case 15 (KU665594), Case 16 (KU665596), Case 17 (KU665595).

values (k:3.333, π:0.00340) when compared with the homologous figures obtained independently from the strains of the nv-Farm (k:2.66667, π:0.00153) and the cv-Farm (k:4.667, π:0.00476).

Interestingly, one recombination event (Rm) was detected between nucleotide sites 1272 and 1416, based on 45 pair-wised comparisons of the 7 complete sequences. When analysed independently, no recombination events were detected in any of the 2 sub-populations of sequences.

As expected, at the polypeptide level the variability was lower. All strains originating from the 2 farms exhibited a common polymorphism involving Ile_{347}, not shared by the 2 vaccines.

DISCUSSION

RHDV2 RNA was detected in most of the rabbits analysed as 18 (87.5%) of the 21 animals investigated were RHDV2-positive. Vaccine RNA was not identified in any of the tissue samples from which viral RNA was amplified and sequenced. Field strains showed clear nucleotide discrepancies with regard to the sequences of the 2 inactivated vaccines (Carvalho *et al.*, 2017) used. None of the 21 rabbits was positive to classic RHDV, in consonance with the notion that RHDV2 has been replacing the classic RHDV strains in wild and domestic rabbit populations of the Iberian Peninsula (Lopes *et al.,* 2015).

The majority of the dead animals did not show any clinical sign of illness and were in good body condition at the necropsy, which is consistent with an acute disease and sudden death. This finding contrasts with other reports stating that RHDV2 induces a more prolonged disease when compared to RHDV (Le Gall-Reculé *et al.,* 2013; Puggioni *et al.,* 2013), resulting in progressive but extensive liver damage that precedes death. The high level of pathogenicity of the circulating strains is also supported by the elevated mortality rates observed in adults (30-40%) and kits (80%), clearly above the values reported previously in RHDV vaccinated/RHDV2 non-vaccinated farms (up to 20% for adult and 50% for kits (Dalton *et al.,* 2012)).

The macroscopic lesions showed that the lungs and liver were the most affected organs, also in agreement with earlier descriptions (Dalton *et al.,* 2012; Duarte *et al.,* 2015a; Lopes *et al.,* 2015).

Pasteurella multocida, a gram-negative, non-motile *Coccobacillus,* was isolated from the lungs of a RHDV2-negative multiparous doe (case 4). Pasteurellosis, a highly contagious disease transmitted either by direct contact or by aerosols, was not detected in any of the other victims, indicating that this disease was neither related to the serial deaths, nor spread through the rabbitry. The source of this single case was not identified.

No possible cause of death was recognised for the other 2 RHDV2-negative rabbits (cases 14 and 20, Table 1), as none of the tested pathogens was detected.

Given that the disease was detected 1 wk earlier in the nv-Farm, it is likely that the virus spread from there into the neighbouring cv-Farm. The proximity and the sharing of some employees most likely contributed to the dissemination of the virus from the nv-Farm into the cv-Farm. The 100% similarity between the 2 strains obtained in October 2014, when the first cases occurred (case 0 from the nv-Farm, case 1 from the cv-Farm, Table 2), provides molecular evidence of an epidemiological link between the 2 events. This is also corroborated by the lack of diversity between these 2 strains and the strains that circulated in both farms between January and April 2015 (99.69 to 99.90% nucleotide similarity), as well as by the reduced variability observed amongst the strains that circulated later in the farm in 2015 (similarities ranging from 99.43 to 99.94%). The nucleotide variability observed (<0.57%) is below the average value (1.3%) previously described among strains originating from different farms (Le Gall-Reculé *et al.,* 2013). The genetic diversity (π) found among nv-Farm strains (0.00153±0.00044) was significantly lower than in the industrial cv-Farm strains (0.00476±0.00192). This may be related to the higher livestock density in the latter, which favours a higher transmission rate, resulting in a faster accumulation of mutations. The 8 strains did not exhibit any unique or exclusive amino acidic variation that could be clearly linked with its putative higher virulence. However, all of them share an Ile at position 347, located in the hyper variable region V2 defined by (Wang *et al.,* 2013). Both vaccines present a different residue at this position. In the large majority of the RHDV2 strains characterised so far, a Thr was mapped at position 347, though a Val or an Ala have also been identified. It is interesting to notice that Ile_{347} is also present in other Portuguese strains previously characterised as originating in the north, centre and south

of mainland Portugal, as well as in the Azores (Duarte *et al.*, 2015a). Most of these strains originated on rabbit farms (KJ683896 and Carvalho *et al.*, unpublished results), but no data was available on the mortality rates induced.

A surprising finding of this study was the death of RHDV2-positive animals that had been vaccinated twice (case 7, Cq 15.27 and 8, Cq 29.08, Table 1), indicating that those animals developed the disease despite vaccination. On the farm, the cold chain for the vaccines is carefully maintained in order to guarantee its preservation. Moreover, all vaccination procedures are carried out systematically by the veterinary assistant according to the protocol, ensuring that all adult animals are vaccinated and that the correct vaccine dose is administrated. This greatly reduces the possibility that lack of vaccination or reduced vaccine dose may have been at the origin of the mortality of the vaccinated does. Individual variability to vaccination may have been at the source of those unexpected outcomes. In view of the lower mortality rate induced by RHDV2 when compared to RHDV, detection of viral RNA in apparently healthy animals that recovered from the infection is expected, as genomic RNA or RNA fragments are known to persist for at least 15 wk after experimental infection (Gall *et al.,* 2006*,* 2007). However, the Cq values of these 2 animals are substantially reduced and too low to represent leftovers from previous infections when compared to the range previously described for RNA and DNA viruses, namely for the blue tongue virus (BTV) (De Leeuw *et al.,* 2015; Barros *et al.,* 2007) and parvoviruses (Duarte *et al.,* 2013). As no animals were sacrificed nor *in vivo* experiments carried out to determine the infectious (viable viral particles) or non-infectious (RNA segments) nature of the RHDV2-RNA, this aspect was not clarified in this study. However, interestingly, they showed no lesions in the liver and lungs and generated a high Cq value (36.14).

The large majority of the RHDV2 RNA-positive rabbits (17/18, 94.4%), including those with Cq values above 30.0 (cases 2, 5, 6), showed typical RHDV2 macroscopic lesions. This fact, along with negative results to other common viral and bacterial pathogens, strongly suggests that all these fatalities, with the exception of case 4, were RHDV2-related.

The death of vaccinated adults indicates that the infection of these animals occurred before an effective immune response could be established, or alternatively, that an ongoing infection hampered the development of an effective immune protection. Exposure to a highly infectious dose, before the vaccine derived protective immune response was fully established, may explain disease development in the adult cases 3 and 7. Despite the virus being able to infect a few vaccinated rabbits from the farm, it did not induce clinical disease in most of the adults that died. Macroscopic lesions were observed in most of the animals but, as the majority of the organs were received frozen, it was neither possible to investigate if typical RHDV2-microscopic lesions were also present in these animals nor to establish a relationship between histo-pathological lesions, viral charge and death for all the animals. This would have been particularly relevant to evaluate the cause of death of the does with lower viral charges.

The significant decrease in the mortality rate observed in the adults after RHDV2 vaccination, which was null after August 2015, compared with the mortality rates observed prior to vaccination (30-40%), exemplified the success of vaccination in controlling the disease.

In the RHDV2 infected kits, a positive association was observed between viral load and the severity of the characteristic anatomo-pathological lesions found in the liver and lungs. Lower Cq values were observed in vaccinated kits (Table 1), suggesting the interference of maternal antibodies with vaccination success, which may have facilitated disease progression (Carvalho *et al.*, 2017).

The time gap between vaccination and casualty varied between 15 d (cases 7, 8, 9, 10 and 11, Table 1) and 121 d (cases 5 and 6, Table 1). Curiously, no trend could be observed between the time gap between vaccination and the Cq value. While no obvious explanation was identified for the lower value of case 3 (Cq 9.53), physiological stress due to recent partum and lactation may have accounted for the progression of the infection in female 7 (Cq 15.27).

The molecular investigation revealed that RHDV2 continued to circulate in the premises for a few months after vaccination. This could be related to the fact that, at any specific time interval, there was always a subset of unvaccinated kits more susceptible to the infection. Infected kits were mostly the offspring of primiparous females, which showed an energy deficit due to simultaneous pregnancy, milk production, and growth, and so were expected to pass lower limited immunity to their litters.

Also interesting was the detection of RHDV2-RNA in an early stage foetus from a RHDV2 positive-doe that had been vaccinated twice, the last shot administered 15 d before death (*results not shown*). A Cq value of 34.08 was obtained from the foetus (*result not shown*), higher than the Cq value (29.08) of the mother, which may suggest the RHDV2 is able to cross the placenta. However, further investigation is required as well as confirmation to rule out foetus contamination with the maternal blood during the uterus opening. To our knowledge, there are no previous reports supporting the congenital infection by RHDV (Xu, 1991).

Neither *Salmonella*, VTEC, *Yersinia*, *Staphylococcus* nor *Listeria* were detected, attesting good sanitary conditions in the rabbitry.

Several risk factors for the introduction of RHDV2 in the rabbitry were identified. The rural surroundings of both farms may have favoured RHDV2 introduction through indirect contact with infected wild species, namely wild rabbits, as no specific protection nets are used. Between 2013 and 2015, a particularly high mortality rate was observed in wild rabbits from legal hunting parks (Dr Fidélia Aboim-Municipal Veterinarian, *personal communication*), the nearest located only 5.3 km away from the farms. According to what has been described in many other regions of the country since 2012 (Abrantes *et al.,* 2013; Lopes *et al.,* 2015; Duarte *et al.,* 2015b), RHDV2 was most probably at the origin of casualties in the wild rabbit population. Therefore, it is possible that RHDV2 transmission occurred from the infected hunting populations into the nv-Farm, via fomites, by human means or arthropods. It has been demonstrated that mosquitoes from the *Culex* genus, active in Portugal from spring to autumn (Alves *et al.,* 2014), play a role in disease dissemination (McColl *et al.,* 2002). Furthermore, the climatic conditions of the area, which has high levels of humidity, create a suitable mosquito habitat.

Likewise, the role of rodents as viral carriers has been supported by the detection of RHDV2 in mammals other than rabbits (Merchán *et al.,* 2011). As the farm is located on agricultural land where rodents are abundant, it is also important to consider their potential role in the transmission of RHDV2 into the rabbitry, although rodent prophylactic measures are carried out.

Another critical point relates to the high resistance of the virus to environmental conditions (Henning *et al.,* 2005). The use of transport vehicles often shared by different rabbit farms may have also accounted for the spreading of the disease locally and across confluent districts.

CONCLUSIONS

The low nucleotide diversity per site (π:0.00340±0.00132) amongst the *vp60* sequences from strains obtained over a 6 mo-period was consistent with a common viral source for the 2 farms. The absolute identity between the strains obtained in both farms during the initial focuses (KU665601 and KU665600) led to recognition of the nv-Farm as the probable source of the virus for the cv-Farm.

Whereas a molecular epidemiological link was established between the 2 farms, no path could be identified as the most probable means for the introduction of the virus in the nv-Farm. However, several risk factors were recognised, relating to the agricultural land where the farms are located, the high mortality of wild rabbits in hunting parks in the same geographic area and the sharing of slaughterhouse vehicles by the rabbitries in the region.

Several months passed before mortality decline was observed following the initiation of vaccination. The time taken before herd immunity could be established undoubtedly highlights the importance of vaccination prior to infection. Several factors may have accounted for the difficulty in reducing and eliminating the virus circulation from the premises, such as the continuous and rapid turnover of the population on intensive farms, which ensures the constant availability of susceptible kits from each new productive cycle, facilitating viral persistence. The putative higher virulence of the strains, supported by the mortality rates observed prior to vaccination, might have required higher antibody titres in the population to impede transmission between animals. No data is known about the immunogenicity of inactivated RHDV2 vaccines when applied as a therapeutic tool to infected populations. However, we believe this dynamic investigation provides preliminary data on the usefulness of vaccination post-infection. While several of these aspects need to be further elucidated, vaccination was proven as an important preventive measure against RHDV2 infection, before rabbitries at risk face this infection.

Acknowledgments: The authors would like to thank Dr. Fidélia Aboim (Municipal veterinarian) for gathering information on the mortality of wild rabbits in several legal hunting parks and to Maria João Teixeira, Fátima Cordeiro and Ricardino Ferreira for their technical assistance. This study was partially funded by a grant from the Fundação para a Ciência e Tecnologia (FCT) SFRH/79225/2011.

REFERENCES

Abrantes J., Lopes A.M., Dalton K.P., Melo P., Correia J.J., Ramada M., Alves P.C., Parra F., Esteves P.J. 2013. New variant of rabbit hemorrhagic disease virus, Portugal, 2012-2013. *Emerg. Infect. Dis., 19: 1900-1902. doi:10.3201/eid1911.130908*

Alves M.J., Zé-Zé L., Amaro F., Osorio, H. 2014. REVIVE 2014 Culicídeos. In Relatório REVIVE 2014 - Culicídeos e Ixodídeos: Rede de Vigilância de Vetores. *Lisboa: INSA IP, 2015. http://hdl.handle.net/10400.18/3026.*

Baily J.L., Dagleish M.P., Graham M., Maley M., Rocchi M.S. 2014. RHDV variant 2 presence detected in Scotland. *Vet. Rec., 174: 411-411. doi:10.1136/vr.g2781*

Bárcena J., Guerra B., Angulo I., González J., Valcárcel F., Mata C.P., Castón J.R., Blanco E., Alejo A. 2015. Comparative analysis of rabbit hemorrhagic disease virus (RHDV) and new RHDV2 virus antigenicity, using specific virus-like particles. *Vet. Res., 46:106. doi:10.1186/s13567-015-0245-5*

Barros S.C., Ramos F., Luís T.M., Vaz A., Duarte M., Henriques M., Cruz, B., Fevereiro M. 2007. Molecular epidemiology of bluetongue virus in Portugal during 2004-2006 outbreak. *Vet. Microbiol., 124: 25-34. doi:10.1016/j.vetmic.2007.04.014*

Calvete C. Sarto P., Calvo A.J., Monroy F., Calvo J.H. 2014. Letter - Could the new rabbit haemorrhagic disease virus variant (RHDVb) be fully replacing classical RHD strains in the Iberian Peninsula?. *World Rabbit Sci., 22: 91-91. doi:10.4995/wrs.2014.1715*

Camarda A., Pugliese N., Cavadini P., Circella E., Capucci L., Caroli A., Legretto M., Mallia E., Lavazza A. 2014. Detection of the new emerging rabbit haemorrhagic disease type 2 virus (RHDV2) in Sicily from rabbit (*Oryctolagus cuniculus*) and Italian hare (*Lepus corsicanus*). *Res. Vet. Sci., 97: 642-645. doi:10.1016/j.rvsc.2014.10.008*

Capucci L., Fallacara F., Grazioli S., Lavazza A., Pacciarini M.L., Brocchi E. 1998. A further step in the evolution of rabbit hemorrhagic disease virus: the appearance of the first consistent antigenic variant. *Virus Res., 58: 115-126. doi:10.1016/S0168-1702(98)00106-3*

Capucci L., Scicluna M.T., Lavazza A. 1991. Diagnosis of viral haemorrhagic disease of rabbits and the European brown hare syndrome. *Rev. Sci. Tech., 10: 347-70. doi:10.20506/rst.10.2.561*

Carvalho C.L., Duarte E.L., Monteiro M., Botelho A., Albuquerque T., Fevereiro M., Henriques A.M., Barros S., Duarte M.D. 2017. Challenges in the rabbit haemorrhagic disease 2 (RHDV2) molecular diagnosis of vaccinated rabbits. *Vet. Microbiol., 198: 43-50. doi:10.1016/j.vetmic.2016.12.006*

Cook H.C. 1997. Origins of ... Tinctorial methods in histology. *J. Clin. Pathol., 50: 716-720. doi:10.1136/jcp.50.9.716*

CRL VTEC. 2013. Identification and characterization of Verocytotoxin-producing *Escherichia coli* (VTEC) by PCR amplification of the main virulence genes. EU Reference Laboratory for *E. coli*, Instituto Superiore di Sanità, Roma. EU-RL VTEC_Method_01_Rev 0: 1-7.

Dalton K.P., Nicieza I., Abrantes J., Esteves P.J., Parra F., 2014. Spread of new variant RHDV in domestic rabbits on the Iberian Peninsula. *Vet. Microbiol., 169: 67-73. doi:10.1016/j.vetmic.2013.12.015*

Dalton K.P., Nicieza I., Balseiro A., Muguerza M.A., Rosell J.M., Casais R., Álvarez Á.L., Parra F. 2012. Variant rabbit hemorrhagic disease virus in young rabbits, Spain. *Emerg. Infect. Dis., 18: 2009-2012. doi:10.3201/eid1812.120341*

De Leeuw I., Garigliany M., Bertels G., Willems T., Desmecht D., De Clercq K. 2015. Bluetongue virus RNA detection by real-time rt-PCR in post-vaccination samples from cattle. *Transbound. Emerg. Dis., 62: 157-162. doi:10.1111/tbed.12100*

Duarte M.D., Henriques A.M., Barros S.C., Fagulha T., Mendonça P., Carvalho P., Monteiro M., Fevereiro M., Basto M.P., Rosalino L.M., Barros T., Bandeira V., Fonseca C., Cunha M.V. 2013. Snapshot of viral infections in wild carnivores reveals ubiquity of parvovirus and susceptibility of Egyptian mongoose to feline panleukopenia virus. *PLoS One 8, e59399. doi:10.1371/journal.pone.0059399*

Duarte M.D., Barros S.C., Henriques A.M., Fagulha M.T., Ramos F., Luís T., Fevereiro M. 2014. Development and validation of a real time PCR for the detection of myxoma virus based on the diploid gene M000.5L/R. *J. Virol. Methods, 196: 219-224. doi:10.1016/j.jviromet.2013.11.014*

Duarte M., Carvalho C., Bernardo S., Barros S.V., Benevides S., Flor L., Monteiro M., Marques I., Henriques M., Barros S.C., Fagulha T., Ramos F., Luís T., Fevereiro M. 2015a. Rabbit haemorrhagic disease virus 2 (RHDV2) outbreak in Azores: Disclosure of common genetic markers and phylogenetic segregation within the European strains. *Infect. Genet. Evol., 35: 163-171. doi:10.1016/j.meegid.2015.08.005*

Duarte M.D., Carvalho C.L., Barros S.C., Henriques A.M., Ramos F., Fagulha T., Luís T., Duarte E.L., Fevereiro M. 2015b. A real time Taqman RT-PCR for the detection of rabbit hemorrhagic disease virus 2 (RHDV2). *J. Virol. Methods, 219: 90-95. doi:10.1016/j.jviromet.2015.03.017*

Duarte M., Henriques M., Barros S.C., Fagulha T., Ramos F., Luís T., Fevereiro M., Benevides S., Flor L., Barros S.V., Bernardo S. 2015c. Detection of RHDV variant 2 in the Azores. *Vet. Rec., 176: 130. doi:10.1136/vr.h497*

Gullett J.C., Nolte F.S. 2015. Quantitative nucleic acid amplification methods for viral infections. *Clin. Chem., 61: 72-78. doi:10.1373/clinchem.2014.223289*

Hall R.N., Mahar J.E., Haboury S., Stevens V., Holmes E.C., Strive T. 2015. Emerging Rabbit Hemorrhagic Disease Virus 2 (RHDVb), Australia. *Emerg. Infect. Dis., 21: 2276-2278. doi:10.3201/eid2112.151210*

Henning J., Meers J., Davies P.R., Morris R.S. 2005. Survival of rabbit haemorrhagic disease virus (RHDV) in the environment. *Epidemiol. Infect., 133: 719-730. doi:10.1017/S0950268805003766*

Hudson R.R., Kaplan N.L., 1985. Statistical properties of the number of recombination events in the history of a sample of DNA sequences. *Genetics, 111: 147-164.*

Le Gall G., Arnauld C., Boilletot E., Morisse J.P., Rasschaert D. 1998. Molecular epidemiology of rabbit haemorrhagic disease virus outbreaks in France during 1988 to 1995. *J. Gen. Virol., 79: 11-16. doi:10.1099/0022-1317-79-1-11*

Le Gall-Reculé G., Zwingelstein F., Laurent S., de Boisséson C., Portejoie Y., Rasschaert D. 2003. Phylogenetic analysis of rabbit haemorrhagic disease virus in France between 1993 and 2000, and the characterisation of RHDV antigenic variants. *Arch. Virol., 148: 65-81. doi:10.1007/s00705-002-0908-1*

Le Gall-Reculé G., Zwingelstein F., Boucher S., Le Normand B., Plassiart G., Portejoie Y., Decors A., Bertagnoli S., Guérin J.L., Marchandeau S. 2011. Detection of a new variant of rabbit haemorrhagic disease virus in France. *Vet. Rec., 168: 137-138. doi:10.1136/vr.d697*

Le Gall-Reculé G., Lavazza A., Marchandeau S., Bertagnoli S., Zwingelstein F., Cavadini, P., Martinelli N., Lombardi G., Guérin J.L., Lemaitre E., Decors A., Boucher S., Le Normand B., Capucci L. 2013. Emergence of a new lagovirus related to Rabbit Haemorrhagic Disease Virus. *Vet. Res., 44: 81. doi:10.1186/1297-9716-44-81*

Le Minor O., Beilvert F., Le Moullec T., Djadour D., Martineau J. 2013. Evaluation de l'efficacité d'un nouveau vaccin contre le virus variant de la maladie hémorragique virale du lapin (VHD). *15èmes Journées de la Recherche Cunicole, 19-20 novembre, Le Mans, France.*

Lopes A.M., Correia J., Abrantes J., Melo P., Ramada M., Magalhães M.J., Alves P.C., Esteves P.J. 2015. Is the new variant RHDV replacing genogroup 1 in Portuguese wild rabbit populations? *Viruses, 7: 27-36. doi:10.3390/v7010027*

Librado P., Rozas J. 2009. DNASp v.5: A software for comprehensive analysis of DNA polymorphism data. *Bioinformatics, 25: 1451-1452. doi:10.1093/bioinformatics/btp187*

Liu S.J., Xue H.P., Pu B.Q., Qian N.H., 1984. A new viral disease of rabbits. *Anim. Husb. Vet. Med.,16: 253-255.*

Marcato P.S., Benazzi C., Vecchi G., Galeotti M., Della Salda L., Sarli G., Lucidi P. 1991. Clinical and pathological features of viral haemorrhagic disease of rabbits and the European brown hare syndrome. *Rev. Sci. Tech., 10: 371-392. doi:10.20506/rst.10.2.560*

McColl K.A., Merchant J.C., Hardy J., Cooke B.D. Robinson A., Westbury H.A. 2002. Evidence for insect transmission of rabbit haemorrhagic disease virus. *Epidemiol. Infect., 129: 655-663. doi:10.1017/S0950268802007756*

McIntosh M.T., Behan S.C., Mohamed F.M., Lu Z., Moran K.E., Burrage T.G., Neilan J.G., Ward G.B., Botti G., Capucci L., Metwally S.A. 2007. A pandemic strain of calicivirus threatens rabbit industries in the Americas. *Virol. J., 4: 96. doi:10.1186/1743-422X-4-96*

Merchán T., Rocha G., Alda F., Silva E., Thompson G., de Trucios S.H., Pagés A. 2011. Detection of rabbit haemorrhagic disease virus (RHDV) in nonspecific vertebrate hosts sympatric to the European wild rabbit (*Oryctolagus cuniculus*). *Infect. Genet. Evol., 11: 1469-1474. doi:10.1016/j.meegid.2011.05.001*

Nei M. 1987. Molecular Evolutionary Genetics. *Columbia University Press: New York.*

Nowotny N., Bascuñana C.R., Ballagi-Pordány A., Gavier-Widén D., Uhlén M., Belák S. 1997. Phylogenetic analysis of rabbit haemorrhagic disease and European brown hare syndrome viruses by comparison of sequences from the capsid protein gene. *Arch. Virol., 142: 657-673. doi:10.1007/s007050050109*

Ohlinger V.F., Haas B., Thiel H.J. 1993. Rabbit hemorrhagic disease (RHD): characterization of the causative calicivirus. *Vet. Res., 24: 103-116.*

Paton A.W., Paton J.C. 1998. Detection and characterization of Shiga toxigenic *Escherichia coli* by using multiplex PCR assays for stx_1, stx_2, *eaeA*, enterohemorrhagic *E. coli hlyA*, rfb_{0111}, and rfb_{0157}. *J. Clin. Microbiol., 36, 598-602.*

Puggioni G., Cavadini P., Maestrale C., Scivoli R., Botti G., Ligios C., Le Gall-Reculé G., Lavazza A., Capucci L. 2013. The new French 2010 Rabbit Hemorrhagic Disease Virus causes an RHD-like disease in the Sardinian Cape hare (*Lepus capensis mediterraneus*). *Vet. Res., 44: 96. doi:10.1186/1297-9716-44-96*

Tham K.M., Barnes S.M., Hunter S.N. 1999. Polymerase chain reaction amplification and gene sequence analysis of a calicivirus from a feral rabbit. *Virus Genes, 18: 235-242. doi:10.1023/A:1008020303036*

Torres J.M., Ramírez M.A., Morales M., Bárcena J., Vázquez B., Espuña E., Pagès-Manté A., Sánchez-Vizcaíno J.M. 2000. Safety evaluation of a recombinant myxoma-RHDV virus inducing horizontal transmissible protection against myxomatosis and rabbit haemorrhagic disease. *Vaccine, 19: 174-182. doi:10.1016/S0264-410X(00)00183-3*

Wang X., Xu F., Liu J., Gao B., Liu Y., Zhai Y., Ma J., Zhang K., Baker T.S., Schulten K., Zheng D., Pang H., Sun F. 2013. Atomic model of rabbit hemorrhagic disease virus by cryo-electron microscopy and crystallography. *PLoS Pathog., 9: e1003132. doi:10.1371/journal.ppat.1003132*

Westcott D.G., Frossard J.P., Everest D., Dastjerdi A., Duff J.P., Choudhury B. 2014. Incursion of RHDV2-like variant in Great Britain. *Vet. Rec., 174: 333-333. doi:10.1136/vr.g2345*

World Organisation for Animal Health (OIE) Technical disease cards. 2015. Rabbit Haemorrhagic Disease. Aetology, Epidemiology, Diagnosis, Prevention and Control References.

World Organisation for Animal Health (OIE), Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2016. Chapter 2.6.2. Rabbit haemorrhagic disease.

Xu W.Y. 1991. Viral haemorrhagic disease of rabbits in the People's Republic of China: epidemiology and virus characterization. *Rev Sci Tech., 10: 393-408.*

Chapter IV

RHD eco-epidemiology

1. Review on RHD eco-epidemiology

1.1 Susceptible host species

The European rabbit has been known as the only species susceptible to RHDV, considered to be highly species specific (Capucci et al., 1996). Both subspecies of the European rabbit, *Oryctolagus cuniculus cuniculus* and *O. c. algirus*, are equally susceptible to RHDV (Abrantes et al., 2013).

The same host specificity was assumed for the European Brown Hare Syndrome Virus (EBHSV) first detected in the early 1980s, prior to the first RHDV outbreak, affecting wild and farmed hares (Capucci et al., 1991)(Wirblich et al., 1994).

However, the many similarities found between RHD and EBHS regarding their clinical presentation and symptomatology, epidemiology, as well as gross pathology and histopathology induced, led many researchers to question the relatedness between both viruses (Capucci et al., 1991). Marcato et al. (1991) even suggested the unifying definition of "infectious necrotic hepatitis of leporids" for the two diseases. In fact, both are characterized by a hyperacute evolution with mild nervous symptoms, degeneration of hepatocytes, necrotic lesions and congestion of the liver, epistaxis, congestion of the spleen and kidneys, presence of uncoagulated blood in body cavities, diffuse or petechial serosal and mucosal haemorrhages and the occasional presence of jaundice (Capucci et al., 1991). Other similarities include the extremely high morbidity and mortality rates, reaching 90% to 100% in adult animals (Wirblich et al., 1994), and enhanced susceptibility of adults, especially breeders, while animals under 40 days of age remain unaffected (Capucci et al., 1991). In addition, the virions of RHDV and EBHSV (both naked virus) are morphologically indistinguishable (Wirblich et al., 1994) and the viral proteins are likewise similar. The two viruses share the same genomic organization in which the genes coding for the non-structural proteins and for the capsid protein (VP60) are part of the same uninterrupted open reading frame (ORF1) (Capucci et al., 1996). Both RHDV and EBHSV are also antigenic related, as demonstrated by Western blot (immunoblot) using hyperimmune serum against RHDV (Chasey et al., 1992)(Wirblich et al., 1994), and neither virus has been adapted to continuous growth in cell culture (Capucci et al., 1991)(Wirblich et al., 1994).

However, discrimination between RHDV and EBHSV was possible by immunoelectron microscopy (Chasey et al., 1992), hemagglutination (Nowotny 1990)(Nowotny, 1991), and enzyme-linked immunosorbent assay (ELISA) (Capucci et al., 1991), showing that the two viruses represent two clearly distinct entities (Capucci et al., 1996) for which both epidemiological features and geographic distribution of outbreaks did not always overlap (Wirblich et al., 1994). For instance,

EBHSV was observed in Scandinavia several years before the first RHD outbreaks (Gavier-Widén and Morner, 1993).
Nevertheless, the remarkable similarities between RHDV and EBHSV prompted several researchers to perform cross-species infections which produced contrasting results (Capucci et al. 1991)(Wirblich et al., 1994). Transmission was reported to be successful in some instances but most cross-species infections have failed to induce disease (Capucci et al., 1991)(Chasey et al., 1992)(Wirblich et al., 1994). Morrise et al. (1991) reported the successfully transmission of EBHSV to rabbits after inoculating them with organ suspensions prepared from EBHSV-positive hares. Also, Di Modugno and Nasti (1990) seem to have transmitted RHDV to two of nine hares which had been inoculated with RHDV-positive rabbit organ suspensions and again reproduced the disease in rabbits, after inoculation with organ homogenates from the two dead hares. In marked contrast, Capucci et al. (1991) were only able to reproduced EBHS in hares and RHD in rabbits.
Recently, Lopes et al. (2014) presented evidences of rabbit lagoviruses cross-species infection through the detection of two classical G1 RHDV strains in two Iberian hares (*Lepus granatensis*) collected dead in Portugal in the 1990s, presenting macroscopic lesions compatible with a lagovirus infection, namely congestion of the liver and lungs, and the presence of non-coagulated blood in the thoracic and abdominal cavities, trachea, lungs and blood vessels. Also, (Lavazza et al., 2015a) demonstrated experimentally that the eastern cottontail rabbit (*Sylvilagus floridanus*) is susceptible to infection with EBHSV.
Cross-species infection was recently described for RHDV2. This new virus is able to infect cape hares (*Lepus capensis*) (Puggioni et al., 2014) as well as Italian hares (*L. corsicanus*), confirming the virus capacity to infect hosts other than rabbits. In Italian hares the virus caused a RHD-like syndrome (Camarda et al., 2014). Recently, this virus was also shown to cause disease in *L. europaeus* (Lavazza A, *personal communication*).

1.2. RHDV survival in the environment

According to Henning et al. (2005), RHDV survival is affected by the duration of exposure and the vehicle used. While virus kept dried on cotton tape (to mimic dried excreted virus in the field) was viable for between 10 and 44 days, virus injected into bovine liver (to mimic RHDV in rabbit carcasses) was still viable after 91 days. These results suggest the virus can survive and remain infectious for at least 3 months in rabbit carcasses in the field, as the surrounding tissue probably protects the virus from desiccation and UV light. RHDV transmission to wild rabbits from

environmental reservoirs is probably influenced by virus source and survival in dead animals' tissues. This could provide a persistent virus reservoir potentially triggering new outbreaks of disease (Henning et al., 2005).
In the laboratory-based studies on RHDV survival carried out by Smid et al. (1991), RHDV in tissue suspensions was shown to survive at 60°C for 2 days (in dried material or in organic suspension) and at 4°C for 225 days (in an organic suspension). Infective particles could be demonstrated in dried material for 105 days at room temperature. Xu (1991) indicated that the virus was viable at least 413 days at -5ºC and 4.5 years at -70ºC lyophilized. Henning et al. (2005) laboratorial experiments on RHDV survival, showed that virus from tissue suspensions dried on cotton tape was able to survive at 60 °C for 2 days and at room temperature for 1–20 days, as confirmed by 100% mortality in inoculated rabbits. However, after 50 and 150 days at room temperature, only one of two rabbits inoculated with the same virus from dried cotton tape succumbed to the disease. These observations were obtained under laboratory conditions, and conclusions for field epidemiology of RHD are limited (Henning et al., 2005). Under laboratory conditions, McColl et al., (2002b) kept rabbit carcasses at 22 °C and collected liver samples up to 30 days *post mortem*. Samples taken up to 20 days *post mortem* were able to infect and kill susceptible rabbits, while those collected after 26 and 30 days did not result in mortality and only some rabbits seroconverted (McColl et al., 2002b). These results suggest that RHDV infectivity decreases over time and are in agreement with Henning et al. (2005) field observations (McColl et al., 2002b)(Henning et al., 2005). However, sample processing in McColl et al. (2002b) experiments might have affected the concentration of infectious RHDV used and resulted in failure to kill susceptible animals after 20 days. RHDV was shown to be resistant to pH=3.0 during 60 minutes (Xu and Chen, 1989) as well as 1h at 37°C or 12h at 4ºC in 0.4% formaldehyde (Smid et al., 1989). However, the virus was shown to be inactivated when submerged 3 hours at 37ºC or 3 days at room temperature in 0.4% formaldehyde (Smid et al., 1989).

1.3. Impact of climate and environmental factors on RHD outbreaks

Seasonal patterns of RHD epidemics are observed, suggesting that some environmental or climatic conditions could trigger an RHD outbreak (Cooke et al., 2002)(Henning et al., 2005). These seasonal patterns may reflect differences in virus behaviour or survival (Cooke et al., 2000)(Cooke et al., 2002).

In Europe, the initial RHD impact on wild rabbit populations appeared to have been strongly influenced by geography and climate, with the greatest declines in rabbit abundance occurring in Spain, Portugal and France (Cooke et al., 2002). In Spain most outbreaks were found to occur in winter or spring (Cooke et al., 2002). A similar pattern was observed in Australia (Cooke et al., 2002). In the most favourable areas for rabbits, generally warmer with annual precipitation of 450 mm – 500 mm, rabbit numbers had a small tendency to recover from RHD outbreaks (Cooke et al., 2002). In marked contrast, in unfavourable areas, populations hardly recovered (Villafuerte et al., 1995)(Cooke et al., 2002).

In addition to virus survival, weather conditions also influence rabbit breeding, rabbit behaviour and the abundance of flying insects which are all interfering factors in RHD outbreaks (Henning et al., 2005).

1.4. RHD Transmission

The natural spread of RHD virus can occur either direct or indirectly (Ohlinger et al., 1993). RHD can be transmitted by direct rabbit-to-rabbit contact (with diseased rabbits or rabbit carcasses). The virus is present in all secretions and excretions of infected rabbits (Ohlinger et al., 1993) such as urine, saliva, nasal, eye secretions and sexual fluids during matting (Cooke et al., 2002). Also, the virus can be transmitted indirectly by mechanical vectors such as insects (Asgari et al., 1998)(McColl et al., 2002a), seabirds (Cooke et al., 2002)(MCcoll et al., 2002a) and rodents (Merchán et al., 2011) or by fomites including hay, food, bedding or contaminated burrows, water, clothing, shoes, cages, equipment and persons (Erber et al., 1991). The latter are especially important in small extensive, non-commercial holdings (Cooke et al., 2002).

Chronically diseased individuals shed infective virus for several weeks after infection (Shien et al., 2000).

Although Argüello-Villares et al. (1988) suggested the RHD airborne transmission, reporting the development of RHD symptoms in rabbits sharing the same area but not the same cage with infected rabbits, no evidences of RHD airborne transmission were shown in either Henning et al. (2005) or Gehrmann and Kretzschmar (1991) work, which were not able to develop RHD symptoms nor RHDV antibodies in control rabbits kept in fly-free room at 50-cm distance from RHDV inoculated animals.

In nature, the faecal-oral route of transmission is probably the most important (Morisse et al., 1991)(Ohlinger et al., 1993), although disease can also be originated by the oral, nasal or parenteral

(i.m. or i.d.) routes (Argüello-Villares et al., 1988)(Henning et al., 2005). Faeces from surviving rabbits can be infectious for susceptible animals up to four weeks after infection (Gregg et al., 1991)(Ohlinger et al., 1991). In Europe, the initial RHDV spread to wild rabbit populations was closely related with the transmission among commercial rabbitries (Cooke et al., 2002). Waste disposal from rabbitries and fresh cut herbage (green feed) used to feed domestic rabbits provided routes for RHDV spread in both directions between wild and captive rabbits (Ohlinger et al., 1993)(Cooke et al., 2002). This contrasted with the situation observed in Australia, where the domestic rabbit industry is small and was not associated with the RHDV initial spread (Cooke et al., 2002).

1.4.1 The role of insects

Insects are known to act as mechanical vectors in the RHD transmission (Barratt et al., 1998)(Crosby and McLennan, 1996). Flies and mosquitoes have been suggested to be involved in RHD long-distance spread (Asgari et al., 1998)(Cooke et al., 2002)(McColl et al., 2002a). RHDV was detected by molecular methods in both flies and mosquitoes (Asgari et al., 1998)(McColl et al., 2002a) and its' persistence in flies that feed on rabbit carcasses could provide an alternative mechanism for viral maintenance in an ecosystem between consecutive epidemics (Henning et al., 2005).

Laboratory work indicated that the larger blowflies (*Caliphora dubia*, *Phormia* sp.) could be potential mechanical vectors implicated in the virus dispersal in Australia (Gehrmann and Kretzschmar, 1991)(Cooke et al., 2002)(McColl et al., 2002a), with contact transmission being the most probably route of infection (Asgari et al., 1998)(Cooke et al., 2002). Blowflies can retain RHDV in the gut for up to nine days after feeding on RHD-infected rabbits (Cooke et al., 2002). Oral and/or anal excretions of flies (flyspots) were found to contain viable virus and constituting a major potential source of the virus for oral or conjunctival transmission to rabbits (Asgari et al., 1998).

Also bushflies, in particular *Musca vetustissima*, were shown to be involved in transmission, potentially transmitting RHDV between rabbits directly or indirectly through contaminated flyspots (Asgari et al., 1998)(McColl et al., 2002a). Bushflies feed naturally on both live and dead animals and are able to penetrate 3–4 layers of cells with their mouthparts, regurgitate during or after biting (McColl et al., 2002a). Given that bushflies can move 7–15 km per day, some authors suggested their role in RHDV spreading in Australia, from the Wardang Island to mainland but also to more distant places within mainland (McColl et al., 2002a).

Lucilia sericata and *Calliphora vicina* were among the species for which RHDV was regularly detected by RT-PCR (Cooke et al., 2002).

Apart from flies, RHDV was also detected in mosquitoes (McColl et al., 2002a). *Culex annulirostris* was shown to transmit RHDV under laboratory conditions (Lenghaus et al., 1994)(Cooke et al., 2002), supporting a role for mosquitoes in the epidemiology of RHD (McColl et al., 2002a). Also, under laboratorial conditions some fleas species, such as *Spilopsyllus cuniculi* and *Xenopsylla cunicularis*, were able to transmit the RHDV, and may also act as mechanical vectors in disease propagation (Lenghaus et al., 1994)(McColl et al., 2002a)(Cooke et al., 2002).

1.4.2 The role of rabbit-sympatric mammalian species

Some micromammals' species are reservoirs and potential sources of RHDV (Merchán et al., 2011). Indeed, RHDV was detected in the liver of rabbit-sympatric micromammals, such as *Mus spretus* and *Apodemus sylvaticus*, demonstrating the capacity of RHDV to infect other species (Merchán et al., 2011). The infected specimens were apparently healthy suggesting that the infection did not lead to disease, but the presence of microscopic lesions was not confirmed (Merchán et al., 2011).

Other evidences of the capability of RHDV to infect a wider broad of hosts came from serological surveys reporting the detection of RHDV antibodies in red foxes and scavengers living in sympathy with RHDV infected rabbit populations (Leighton et al., 1995)(Frölich et al., 1998)(Parkes et al., 2004). This data may imply other species in the epidemiology and persistence of the disease (Merchán et al., 2011).

1.4.3. The putative role of vehicles and human movements in the long distance spread of RHDV

It is known that, in the swine industry, the contamination of transport vehicles and equipment used to move pigs from farms to harvest facilities most likely plays a role in the rapid dissemination of Porcine Epidemic Diarrhea Virus (PEDV) between farms and across vast geographic regions (Lowe et al., 2014). Also, a potential route of porcine reproductive and respiratory syndrome virus (PRRSV) transmission is the contaminated transport vehicle (Dee et al., 2004). Although, to our knowledge, there is no study on the risk of RHDV spread by transport vehicles, there is the same kind of concerns regarding transport by the veterinarians of the rabbit industry (Dr. José Monteiro, *personal communication*). In addition, as for swine (Lowe et al., 2014), transport vehicles are often shared by different rabbitries, favouring the spread of diseases (Dr. José Monteiro (Coren), *personal communication*). However, it is noteworthy that the "*all in–all out*" system used in the swine

industry (Lowe et al., 2014), as well in modern rabbitries (Dr. José Monteiro (Coren), *personal communication*), may limit the spread of disease due to contaminated transport vehicles (Lowe et al., 2014).

It is also a fact that human movements have played an important role in infectious diseases dissemination and that it will continue to shape the emergence, frequency, and spread of infections in new geographic areas and populations (Wilson, 1995). The global movement of humans and materials and the concomitant changes in the environment, climate, technology, land use, human behaviour, and demographics converge to favour the emergence of infectious diseases caused by a broad range of organisms in humans, plants and animals (Wilson, 1995). Recent phylogenetic studies within the frame of this thesis (Chapter V, **Studies 8 and 9**) clearly showed the importance of human movements in the emergence of RHDV2 in the Azores and Madeira archipelagos.

1.5. The effect of rabbit population dynamics on RHD impact

The initial impact of RHD was greater in high-density rabbit populations of Spain, Portugal and Australia (Cooke et al., 2002)(Calvete, 2006), probably because high densities of susceptible rabbits favoured the transmission of the virus (Calvete, 2006).

As the disease became enzootic, many populations continued to decrease and even became extinct (Calvete, 2006). However, despite the rapid transmission of RHDV in the Iberian Peninsula, five years were required for RHDV to reach most wild rabbit populations in Spain and a similar scenario was observed in France (reviewed in (Cooke et al., 2002)). Also, not all rabbit populations were affected when the disease emerged and some hunting reserves remained untouched (Cooke et al., 2002). In addition, some rabbit populations made better recoveries than others (Calvete et al., 2006). In Europe, the effect of RHDV on rabbit abundance showed a north-south gradient with the greatest declines recorded in Portugal and Spain (Calvete, 2006).

To respond to the paradox of how RHDV caused huge mortality in some populations apparently persisting in others at high prevalence in the absence of disease, a few models have been created by a few research groups (White et al., 2002)(Calvete et al., 2006)(Fouchet et al., 2009).

Some modelling studies on RHD, have explained the reduced impact of the disease in some populations as well as the differential impact of RHD along the north-south gradient in Europe with the existence of non-pathogenic protective RHDV-*like* viruses or pathogenic RHDV (White et al., 2002). According to White et al. (2001) avirulent strains of RHDV could induce long lasting disease to balance their poor level of transmission. The White et al. (2002) model introduced the concept

that RHDV has two modes of transmission, that compete simultaneously for susceptible hosts, and seem to clarify the paradox of how RHDV can be highly prevalent in some populations, but with low or no mortality. According with White et al. (2002), a highly contagious and virulent pathogen may also utilize this avirulent alternative transmission mode. They showed that the basic reproductive number of avirulent strains depended on the life expectancy of rabbits, which varies between populations (White et al., 2001). In accordance, each strain could be locally selected in some areas but not in others and the diffusion of the virus between populations may explain their coexistence (White et al., 2001). The differences in host demography in the model determine whether avirulent transmission prevents large-scale mortality (as in most United Kingdom populations) or not (White et al., 2002).

The Fouchet et al. (2009) model allows testing demographic (birth rate, host subpopulation size, level of connectivity between subpopulations), epidemiologic (transmission rate) and genetic (of both hosts and parasites) characteristics of the host-parasite interaction. According to Fouchet et al. (2009) despite their strong competitive advantage, highly virulent strains are unadapted to small subpopulations that go extinct rapidly, limiting the virus chances of colonizing other subpopulations. These strains seem to be better local competitors and are favoured when pathogen exchanges between subpopulations are more frequent. The trade-off between the persistence of the virus within subpopulations and its capacity to colonize other subpopulations leads to the selection of intermediate virulent strains, depending on several factors. When a factor favours the persistence of all strains, selection seems to favour more transmissible but also more virulent strains. In particular, a high birth rate, a low virus induced mortality, a high connectivity between subpopulations, a low local transmission rate and very large and very small host subpopulations favour highly virulent strains. On the contrary, genetic diversity, by decreasing the size of the host population available for each strain, generally selects for less virulent strains. The Fouchet et al. (2009) model also allows determining how strains features interplay with the subpopulation characteristics. In particular, a feedback exists between pathogen evolution and host demography and strains that invest in persistence (*i.e.* transmission over long periods by infected individuals) have no advantage in very small subpopulations. The differential success between virulent and avirulent strains depends on rabbit population characteristics (e.g., size, connectivity). The fact that no RHDV strain of intermediate virulence has been isolated in the field which is in contradiction with the Fouchet et al. (2009) model prediction of selection of intermediate virulent

strains, and can be justified by the physiological reason that makes RHDV either benign or highly lethal.

Calvete et al. (2006) evaluated the impact of RHD on rabbit populations with a simple, age-structured deterministic model that considered the existence of a unique pathogenic RHDV with a unique mode of transmission. These authors based their model in the observation that in most favourable areas for rabbits before RHD spread, there was a clear tendency for rabbit numbers to recover in geographically limited populations. Also, in Iberian wild rabbit populations, recovery from RHD was improved in the most suitable habitats (Villafuerte et al. 1995). According to the model, RHD could be substantially decreased by managing rabbit populations and RHD dynamics, principally by increasing the carrying capacity of the habitat and the productivity of rabbit populations in those areas where environmental conditions are favourable for the species. The long-term stable recovery of rabbit populations should hence be mainly based on improving habitat suitability (Calvete et al., 2006). Also, according to this model, when low density populations show an increased density, the impact of RHD raises substantially before the populations can reach densities at which disease impact starts decreasing. During this transitional process, other events affecting rabbits, such as flooding, hunting pressure, predation impact or other infectious diseases (e.g. myxomatosis), may slow rabbits recovery or even cause the extinction of rabbit populations.

In brief, according to Calvete et al. (2006) the long-term impact of RHD is conditioned by population dynamics, in turn determined by habitat suitability and partially by climatic conditions. In accordance, the RHDV impact should be lower in populations located in the most suitable habitats.

1.6. Management strategies to enhance wild rabbits' populations

Most Iberian rabbit populations are still declining and several management techniques have been employed to reverse this scenario and enhance wild rabbit populations' growth for conservational purposes. Management actions for the wild rabbit aim at minimizing the impact of adult and juvenile high mortality, caused by viral diseases or predation, and incrementing the productivity of the population (e.g. warren building, food) (Ferreira and Delibes-Mateos, 2010). Population monitoring, adjusting hunting pressure, predator control, habitat management, restocking and rabbit vaccination are the most frequently employed management tools (Calvete and Estrada, 2004)(Ferreira and Delibes-Mateos, 2010).

Evaluating the impact of these management techniques includes assessing basic biological parameters from natural populations, the use of standardised rabbit monitoring protocols producing systematic and periodic comparable results, assessing the impact of predator control, the costs/benefits of wild rabbit vaccination and the effectiveness of habitat management (Ferreira and Delibes-Mateos, 2010).

In Portugal and Spain, conservation strategies of wild rabbit populations have usually been based on restocking operations and habitat management (Ferreira and Alves, 2009). These efforts have not been followed by an increase in rabbit population numbers, possibly as a consequence of lack of funds to maintain habitat management, high mortality associated to new disease outbreaks and poor knowledge of the most suitable places to introduce farmed raised animals (Ward, 2005)(Ferreira, 2012)(Guerrero-Casado et al., 2013)(Ferreira and Ferreira, 2014).

1.6.1 Population monitoring programs

The establishment of a long-term programme using a standardized methodology for large-scale monitoring of rabbit abundance and trends in the Iberian Peninsula is imperative to understand the extent and causes of rabbit population declines at the Iberian level (Ferreira and Delibes-Mateos, 2010).

Under the frame of the Wild Rabbit Recovery Programme (PRECOB – programa de recuperação do coelho-bravo; Official Portuguese Governmental Journal 296/2007, 8-01), a national wild rabbit monitoring methodology and network named "INCOB" was created and implemented in the field. As reviewed by Ferreira and Delibes-Mateos (2010), this network used a standardised data gathering system on population parameters in areas where rabbits are keystone species. The system consists of a stratified sampling of four 250 m fixed linear transects along which rabbit signs are counted (latrines mainly), ideally twice a year (late spring and late autumn) and distributed over 2x2 Km UTM square units.

1.6.2 Hunting pressure

Game management is one of the most readily available tools for rabbit recovery in hunting reserves and it may involve adjusting hunting pressure (e.g. hunting days, number of hunters) and hunting bags (number of rabbits harvested) (Ferreira and Delibes-Mateos, 2010). These adjustments are frequently made by hunters on their own initiative (Ferreira and Delibes-Mateos, 2010). In northern Spain, recovering rabbit trends were positively correlated with low hunting pressure (Williams et al.,

2007). Nevertheless, these adjustments on hunting pressure *per se* are insufficient to assure rabbit recovery (Ferreira and Delibes-Mateos, 2010).

1.6.3. Predator control

Legal predator control is commonly used throughout the Iberian Peninsula to control overabundant game predator species that can potentially inflict damage in preys (Ferreira and Delibes-Mateos, 2010). However, predator control poses a serious threat to biodiversity, as frequently there is no true knowledge of the real predator population size or on the extent of the damages inflicted in prey populations, but also because it may affect target and non-target species (Ferreira and Delibes-Mateos, 2010).

Currently, predator control is often associated with rabbit translocations, as it is believed that preventing predator activity can substantially increase rabbit survival during restocking efforts (Calvete et al., 1997).

Overall, there is little information on the impact of this technique on target and non-target species in the Iberian Peninsula or even whether it accomplishes its final goal (Ferreira and Delibes-Mateos, 2010).

1.6.4. Habitat management

As reviewed by Ferreira and Alves (2009), habitat management has become one of the most commonly used measures to restore wild rabbit populations by improving basic ecological resources availability, habitat quality and the carrying capacity of a given area. These measures are considered to be effective in Mediterranean ecosystems. The technique is generally low cost and of simple application while not inducing short- or long-lasting negative biological effects in native populations. Habitat management has a global positive impact on biodiversity by beneficiating both target and several other species.

In areas with low rabbit densities, habitat management artificially mimics the species' ability to impose structural alterations in the environment when abundant (Ferreira and Alves, 2009). These measures aim at improving global habitat quality, strongly influencing population survival and the success of other management techniques, such as restocking (Ferreira and Alves, 2009). Habitat management may include the improvement of shelter conditions to promote a quantitative and qualitative increase of breeding sites and refuge cover from predators (Ferreira and Delibes-Mateos, 2010). These measures also comprise providing additional high quality food sources closer

to shelter patches through the establishment of pastures/crops or alternatively clearing scrubland (Ferreira and Delibes-Mateos, 2010).

Habitat management techniques are widely used by game managers in hunting reserves in the south of Portugal as one of the first approaches to recover wild rabbit populations (Ferreira and Alves, 2009).

1.6.5. Translocations/Restocking and captive breeding in semi-extensive enclosures

Although common measures to increase rabbits' populations, rabbit translocations and restocking are usually expensive and with generally low success (Calvete et al., 1997)(Ferreira and Delibes-Mateos, 2010). The most important factors responsible for the failure include handling, capture stress and the impact of a new environment, greatly related to a high mortality rate both from predation and deterioration of the rabbits' physical condition as a consequence of disease (Calvete et al., 1997). The critical adaptation period for restocked rabbits is of approximately 10 days, after which survival rates are greater (Calvete et al., 1997). Longer adaptation periods after rabbit translocation can minimize mortality (Ferreira and Delibes-Mateos, 2010).

Rabbits used for restocking operations are usually wild specimens captured from large natural populations (Calvete et al., 1997). As these animals need to be protected from viral diseases to increase their chances of long term survival in the new environment, the usual transfer protocol includes vaccination and rabbits' immediate release (Calvete et al., 1997).

In recent years, the establishment of semi-extensive rabbit captive breeding enclosures has become widely used within the scope of conservation projects to obtain healthy and genetically pure rabbits for soft release of individuals into the wild (Ferreira and Delibes-Mateos, 2010). In the process, rabbits are allowed to adapt to their release site for a variable time period prior to release. This is an alternative to plain restocking (immediate release of previously captured rabbits from other sites) or translocations (transference of rabbits from one site to the other with or without an adaptation period) (Ferreira and Delibes-Mateos, 2010).

1.6.6. Disease control and vaccination

Vaccination campaigns of wild rabbit populations against RHD and myxomatosis in specific areas are some of the mitigation strategies proposed. Assuming they are effective in increasing antibody titres and, thus, preventing both diseases, they are management tools frequently used in the recovery of wild populations (Cabezas et al., 2006). Usually, rabbits with unknown immunological

status are captured by trapping or ferreting and vaccinated with commercial vaccines against both diseases to be released again ate the capture site. Trapping and handling of young rabbits may increase their mortality rates (Cypher, 1997)(Calvete and Estrada, 2000) and the efficacy of immunisation depends on the vaccine used and the physiological status of the animal (Calvete et al., 2004b). In addition, the proportion of the population that can be captured and vaccinated and the population dynamics are important in determining the efficacy of a vaccination campaign (Calvete et al., 2004b). To be effective, a large fraction of the population needs to be vaccinated every year (Calvete et al., 2004b). The long-term effects of vaccination on wild rabbits' survival have not been examined and information on the efficacy of previous vaccination campaigns is scarce (Calvete et al., 2004b). In Calvete et al. (2004b) study, unvaccinated young rabbits between 180 and 600 g were 13.6 times more likely to die than vaccinated young rabbits. In adults, vaccination was associated with a slight, but statistically non-significant, reduction in the mortality, suggesting that a high level of natural antibodies resulting from exposure to the field viruses overlapped the effects of vaccination.

Another limitation of vaccination of wild rabbits' relates to the fact that inactivated commercial vaccines, designed for domestic rabbits, have been used (Calvete et al., 2004b). A new generation of recombinant vaccines has been developed that are expected to be more effective (Bertagnoli et al., 1996)(Castañón et al., 1999)(Fernández-Fernández et al., 2001)(Bárcena et al., 2000)(Torres et al., 2000), some of which were designed to be naturally transmissible (Torres et al., 2000), and specifically intended to be used in wild populations (see Chapter III, point "2. Review on RHD prophylaxis"). Since its emergence, several vaccines were released into the market for RHDV2 control all to be administrated by parenteral route, therefore with restrict application to wild populations.

Given the alarming impact of RHDV2 in the wild rabbit populations, the Portuguese Government has recently activated a plan aiming the control of the disease (Despatch 4757/2017 of May 31st) that includes several of the management strategies described above.

In this chapter, two studies were included, namely **Study 6**, essentially epidemiological, where we investigated a RHDV2 outbreak in a wild rabbit breeding unit located in Barrancos and **Study 7** that traced the passage of RHDV2 through the Berlengas archipelago from where the rabbit is currently being eradicated.

Study 6

Six years after its emergence, what challenges still poses RHDV2 to the establishment of natural wild rabbit populations?

Tracking the origin of a rabbit haemorrhagic virus 2 outbreak in a wild rabbit breeding centre in Portugal; epidemiological and genetic investigation

Carina L. Carvalho[1], Joana Rodeia[2], Sandra Branco[1,3], Madalena Monteiro[4], Elsa L. Duarte[1,3], Pedro Melo[5], Patricia Tavares Santos[6], Ana M. Henriques[4], Sílvia S. Barros[4], Fernanda Ramos[4], Teresa Fagulha[4], Miguel Fevereiro[4], Margarida D. Duarte[4]

[1]Instituto de Ciências Agrárias e Ambientais Mediterrânicas (ICAAM), Universidade de Évora, Évora, Portugal; [2]Empresa de Desenvolvimento e Infra-estruturas do Alqueva (EDIA), Portugal; [3]Departamento de Medicina Veterinária, Universidade de Évora, Évora, Portugal; [4]Instituto Nacional de Investigação Agrária e Veterinária (INIAV), Lisboa, Portugal; [5]Vetnatura, Portugal; [6]Direção Geral de Alimentação e Veterinária, Oeiras, Portugal;

Journal of Emerging Infectious Diseases
doi1: 114. doi:10.4172/24724998.1000114.

Carvalho et al., J Emerg Infect Dis 2016, 1:4
DOI: 10.4172/2472-4998.1000114

Research Article Open Access

Tracking the Origin of a Rabbit Haemorrhagic Virus 2 Outbreak in a Wild Rabbit Breeding Centre in Portugal; Epidemiological and Genetic Investigation

Carina Luísa Carvalho[1], Joana Rodeia[2], Sandra Branco [1,3], Madalena Monteiro[4], Elsa Leclerc Duarte[1,3], Pedro Melo[5] Patrícia Tavares Santos[6], Ana Margarida Henriques[7], Sílvia Santos Barros[7], Fernanda Ramos[7], Teresa Fagulha[7], Miguel Fevereiro[7], Margarida Dias Duarte[7]

[1]*Institute of Mediterranean Agricultural and Environmental Sciences (ICAAM) and Institute for Advanced Studies and Research (IIFA), University of Évora, Núcleo da Mitra, 7000 Évora, Portugal*
[2]*Enterprise Development and Infrastructure Alqueva (EDIA), Rua Zeca Afonso, nº 2, 7800-522 Beja, Portugal.*
[3]*Department of Veterinary Medicine, School of Sciences and Technologies, University of Évora, Núcleo da Mitra, 7000 Évora, Portugal*
[4]*National Institute for Agricultural and Veterinary Research (INIAV), Pathology Laboratory, Av. da Républica, Quinta do Marquês, 2780-157 Oeiras, Portugal*
[5]*Vetnatura. Calçada Palma de Baixo, 8, 7ºC, 1600-176 Lisboa, Portugal*
[6]*Directorate General of Food and Veterinary (DGAV), Campo Grande 50, 1700-093 Lisboa, Portugal*
[7]*National Institute for Agricultural and Veterinary Research (INIAV), Virology Laboratory, Av. da Républica, Quinta do Marquês, 2780-157 Oeiras, Portugal*

Abstract

As key prey, the wild rabbit downsize constitutes a major drawback on the endangered Iberian lynx (*Lynx pardinus*) re-introduction in the Iberia. Several captive breeding units mostly located in Alentejo, endeavour the wild rabbit repopulation of depleted areas assigned for the lynx re-introduction.

Here we report an RHDV2 outbreak that occurred in early 2016 in a wild rabbit captive breeding unit located in Barrancos municipality. The estimated mortality rate between March and April 2016 was approximately 8.67%. Anatomopathologic examination was carried out for 13 victimized rabbits. Molecular characterization was based on the complete *vp60* capsid gene.

The 13 rabbit carcasses investigated showed typical macroscopic RHD lesions testing positive to RHDV2-RNA. Comparison of the *vp60* nucleotide sequences obtained from two specimens with others publically available disclosed similarities below 98.22% with RHDV2 strains originated in the Iberia and Azores and revealed that the two identical strains from Barrancos-2016 contain six unique single synonymous nucleotide polymorphisms.

In the phylogenetic analysis performed, the Barrancos-2016 strains clustered apart from other known strains, meaning they may represent new evolutionary RHDV2 lineages. No clear epidemiological link could be traced for this outbreak where the mortalities were lower compared with previous years. Yet, network analysis suggested a possible connection between the missing intermediates from which the strains from Barrancos 2013, 2014 and 2016 have derived. It is therefore possible that RHDV2 has circulated endemically in the region since 2012, with periodic epizootic occurrences.

Still, six years after its emergence in wild rabbits, RHDV2 continues to pose difficulties to the establishment of natural wild rabbit populations that are crucial for the self-sustainability of the local ecosystems.

Keywords: Rabbit haemorrhagic disease; RHDV2; Outbreak; Phylogeny; Captive wild rabbits; Iberian lynx; Conservation

Introduction

Rabbit haemorrhagic disease virus 2 (RHDV2) is a small, fast-evolving RNA virus belonging to the *Caliciviridae* family [1].

It is known that RNA viruses' adaptability is the result of their extreme mutation frequency, as many genomic variants are created at a high generation rate [2]. Among them, non-synonymous mutations at one or more sites may alter the RNA virus phenotypes and virulence [3,4]. Indeed in RHDV infections, like for other viruses, antigenic drift (minor changes in surface generated through point mutations) render hosts susceptible if at least a portion of the population has not been previously exposed to the new antigen(s) [5].

The mean evolution rate for RHDV based on the analysis of complete capsid gene sequences was estimated in 5.48 to 7.7 × 10^{-4} substitutions/site/year [6,7]. It has been suggested that the emergence of virulence occurred in the first quarter of the 20th century in a different host until it jumped to *Oryctolagus* spp. in China, where the disease was first reported in 1984 [7-9]. A similar process of lagovirus species jump was proposed for the emergence of RHDV2, although its putative host was not yet identified [8].

Although genetically related with RHDV, RHDV2 emergence in 2012 in Portugal had a tremendous impact in wild rabbit populations and was a major drawback for the re-introduction of the captive-bred Iberian lynx (*Lynx pardinus*), the most endangered wild felid in the world.

***Corresponding author:** Margarida D Duarte, National Institute for Agricultural and Veterinary Research (INIAV), Virology Laboratory, Av. Of the Republic, Quinta do Pombal, 2780-157 Oeiras, Portugal, Tel: +351 214 767 490; Fax: +351 214403666, E-mail: margarida.duarte@iniav.pt

Received September 20, 2016; **Accepted** October 27, 2016; **Published** November 07, 2016

Citation: Carvalho CL, Rodeia J, Branco S, Monteiro M, Duarte EL, et al. (2016) Tracking the Origin of a Rabbit Haemorrhagic Virus 2 Outbreak in a Wild Rabbit Breeding Centre in Portugal; Epidemiological and Genetic Investigation. J Emerg Infect Dis 1: 114. doi:10.4172/2472-4998.1000114

Copyright: © 2016 Carvalho CL, et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Wild rabbits are the main component of Iberian lynx diet and a keystone species on the equilibrium of the Iberian ecosystem [10]. Endemic to the Iberian Peninsula [11], the Iberian lynx population size dramatically decreased during the 20th century [12]. In 2002 was declared 'critically endangered' (CR) by the International Union for the Conservation of Nature (IUCN) [13,14]. Major efforts (Action Plan for the Conservation of the Iberian Lynx in Portugal; Iberian Lynx *exsitu* Conservation Programme; projects under the European LIFE Programme [Lince Moura/Barrancos - LIFE06 NAT/P/000191; Habitat Lince Abutre - LIFE08 NAT/P/000227; Iberlince - LIFE10 NAT/ES/000570], among others) have been made in the last decade to reverse the decline of this species. As a result, in 2015, the Iberian lynx species status was shifted to "Endangered" (EN), due to the increase of the populations in Spain. However, the RHDV2 emergence in Portugal in 2012 almost decimated the entire wild rabbit population in particular niches of mainland [15,16] and Azores [17]. A comparable scenario was also observed in Spain [18]. Although both *Oryctolagus cuniculus* subspecies are equally affected by RHDV2 [15,18], the susceptibility of the subspecies *O. cuniculus algirus* acquires special importance considering its restricted distribution to southwest [15] and the dramatic implications of RHDV2 outbreaks on its frail conservation status [16,18]. *O. cuniculus algirus* is a key prey species for several carnivores, including the Iberian Lynx and the Iberian imperial eagle *(Aquila adalberti),* both emblematic and endangered species in Portugal, and a population downsize may lead to a series of major ecological and economic problems [19].

To counteract the effects of RHDV2 on the decrease of wild rabbit populations in Portugal, several measures were implemented among which restrictive hunting regulations and captive breeding of wild rabbits for subsequent return to wildlife. Rabbits restocking efforts have been recently reinforced to repopulate depleted areas in the sequence of RHDV2 outbreaks as a measure to fasten self-sustainable population establishment. Wild rabbits are breed in captivity units and set free in areas assigned either for cinegetic activity or for the Iberian lynx re-introduction. For the latter purpose, most units are located in Moura and Barrancos municipalities.

Since its first detection in Portugal in 2012 [15], RHD has been observed in some of those wild rabbit breeding units. However, to date, none of these past outbreaks were characterized and publically reported.

Here, we describe an RHDV2 outbreak in a wild rabbit captive breeding unit, built in 2004 for the wild rabbit repopulation of the Noudar Nature Park, one of the possible sites for future conservation translocations of the Iberian lynx. Phylogenetic analyses were carried out to assess the variability and putative relation of two RHDV2 Barrancos-2016 strains with other strains obtained previously in the unit, the first time in late December 2012, as well as with strains from the South, envisaging to unravel possible links between different outbreaks.

Materials and Methods

Samples and inquiry

A total of 13 victimized rabbits collected between March and April 2016, were investigated at Instituto Nacional de Investigacao Agraria e Veterinaria (INIAV). An inquiry was carried out to gather epidemiologic information on the wild rabbit captive breeding unit regarding its exact location and surroundings, extension of the parks, road accesses and animal imports (dates and animal number).

Estimation of density and mortality in 2016

Direct counts are difficult since these animals spend most of the day hidden in their warrens, making indirect counting methods preferable. Rabbits' abundance was therefore estimated based on the intake of commercial dry rabbit food, supplied weekly to each park, assuming it was the main food source and that the average consume per rabbit per week is about 1 kg.

Mortality rates were estimated comparing the animal density inferences, calculated as described above, before and after disease outbreaks. In the unit, enumeration methods [20] (collection of cadavers) are used quite rarely for mortality estimations since they can contribute to bias results and underestimate mortality numbers as a consequence of pre-emergence death (before litters' emerge from the nest) and predation [21].

Anatomopathological examination

Necropsies were carried out at the University of Evora. Liver and lung samples were collected and sent to INIAV for histopathological examination. Samples were fixed in 10% buffered formalin and embedded in paraffin by standard procedures. Five micrometer-thick sections were stained with haematoxylin and eosin (H & E) prior light microscopy examination.

Virological examination

Liver and lungs samples were homogenized with phosphate buffered saline (PBS) and clarified at 3,000 g for 5 min. DNA and RNA were extracted from 200 µl of the clarified supernatant in a BioSprint 96 nucleic acid extractor (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RHDV2-RNA was assessed by RT-qPCR [22] using the One Step RT-PCR kit (Qiagen, Hilden, Germany). Screening for classical RHDV strains was performed by conventional RT-PCR followed by sequencing analysis of the amplicons obtained with primers RC-9 and RC-10 [23], also using the One Step RT-PCR kit (Qiagen, Hilden, Germany). Myxoma virus was investigated by qPCR as described by Duarte et al. [24] with the FastStart TaqMan Probe Master Kit (Roche, Roche Diagnostics GmbH, Manheim, Germany). Cq values above 40 were considered negative.

Sequencing analysis, multiple alignments and genetic distances

Amplification of the full *vp60* sequences was accomplished by two overlapping fragments obtained with primers 27F (5'-CCATGCCAGACTTGCGTCCC-3') and 986R (5'-AACCATCTGGAGCAATTTGGG-3') and with primers 717F (5'-CGCAGATCTCCTCACAACCC-3') and RC10R [23].

Sequencing was carried out using a BigDye™ Terminator cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) and the nucleotide sequences determined on an automated 3130 Genetic Analyzer system (Applied Biosystems, Foster City, CA, USA). The complete *vp60* sequences obtained from two animals (strains Barrancos1/PT16 and Barrancos2/PT16) were submitted to GenBank and attributed the accession numbers KX132812 and KX132813. Nucleotide alignments were performed with Clustal Omega [25].

The percent nucleotide variability among strains based on the *vp60* gene (Table 2) and the genetic divergence between populations, inferred by the nucleotide differences and substitutions between viral populations, was calculated using the DnaSP software (Version 5.10.01) [26], in order to further explore the genetic relationships among

strains. Strains from the same geographic origin and year of collection were included in the same population.

Phylogenetic analysis

The appropriated substitution model was determined resourcing to R software (R Development Core Team, 2009). The GTR model [27] with gamma-distributed rate variation across sites showed the lowest BIC and AICc values and was subsequently used to infer phylogenetic relationships using ML analysis. Robustness of the tree nodes was assessed by bootstrapping 1000 times.

For the Bayesian analysis the CLUSTAL Omega results were converted to the NEXUS format using Mesquite software [28]. The phylogenetic tree was obtained with a Bayesian inference of phylogeny throughout the MrBayes version 3.1.2 software that uses the Markov chain Monte Carlo simulation technique to approximate the posterior probabilities (PP) of trees [29,30]. MrBayes analysis was performed using the GTR model (nst=6) with gamma-shaped rate variation with a proportion of invariable sites (rates=invgamma). The analysis was run for 10^6 generations (ngen=10^6) with four chains of temperature (nchains=4), and each chain was sampled every 10th generations (samplefreq=10).

The graphical representation and edition of the phylogenetic trees were performed with FigTree v1.3.1.

The Network software (version 5.0) was used to reconstruct a network using haplotypes (*vp60* gene) by median joining [31].

Results

Data collected from the inquiry revealed that the unit affected by RHDV2, located in Barrancos municipality near the Spanish border, encompasses one quarantine and four reproduction parks. The five parks are sited in holm oak woodlands, only accessible by sand-clay roads, and lay on a 999 hectare extension area delimited by two riversides: Southeast and within the national territory by the Múrtega River, and Northwest by the Ardila River, in the Spanish border (Figure 1). Parks 1 (P1) and 3 (P3) are located closer to a busy road and therefore more exposed to human activity. In contrast, parks 2 (P2) and 4 (P4) lay on a more protected area, given their proximity to the riverside and, in the P4 case, also to a closed ore mine more distant from the roads.

The first animals (100 adults, 80 females and 20 males from subspecies *O. cuniculus algirus*) were introduced in 2004 (by the time of P1 construction) and originated from captive-bred wild populations from Montemor-o-Novo, located 136 Km northwest. Introduction of additional *O. cuniculus algirus* rabbits from other breeding parks was performed regularly (Table 1). From the second half of 2013 afterwards, following the first RHDV2 outbreak a higher number of foreign bred rabbits, mostly originated from the Spanish Granada Province, were introduced into the parks (Table 1). The last introduction took place in 2015. The actual animal density is estimated in approximately 120 animals per hectare (≈1500 rabbits over a 12.5 hectare area). Rabbits are vaccinated against myxoma virus and classical RHDV and identified with a tattoo or ear tag before release. No other prophylactic measures besides a coccidiostatic administration through the feed are carried out.

Mortality by RHDV2 had been observed in the parks since 2012 (Table 1).

In 2016, the first RHDV2 fatalities occurred in March, affecting rabbits of all ages from P1, P2 and P3. No casualties were registered in P4. Until April 2016, the mortality rate was estimated at approximately 8.67% (130/1500). No more casualties were registered from April onwards.

A total of 13 animals, corresponding to approximately 10% of the estimated casualties, were necropsied and epistaxis was observed in all animals. Haemorrhagic tracheitis, hepatic congestion and friable liver were also observed. In two animals, histopathology revealed congestion of the lungs and necrotic lesions in the liver.

All liver samples (n=13) were positive to RHDV2 by RT-qPCR [22] and negative to myxoma virus by qPCR [24]. Sequencing analysis of *vp60* 386 bp long fragments ruled out the concomitant presence of classical RHDV strains.

The complete *vp60* nucleotide sequences (1740 bp long) obtained from two animals (KX132812 and KX132813) showed to be identical (100% similarity). Comparison with complete *vp60* sequences publically available revelead the presence of six unique single synonymous nucleotide polymorphisms (SNPs) at positions 618, 940, 1020, 1077, 1440 and 1584. Blast analysis (14th June 2016) disclosed a similarity of about 98% with strain RHDV-N11 from Spain (KM878681) as well as with several strains from across Portugal mainland and Azores.

Figure 1: Aerial photo of the unit. The five parks and both rivers that delimit the unit are indicated by white arrows. The border between Spain and Portugal is highlighted by the yellow dashed.

Year	No of Parks in the unit	Translocated animals		No. of animals	Causalities	
		No.	Origin		No. of estimated casualties	Estimated mortality rate
2009	2	55	PT mainland	unknown[c]	unknown[c]	unknown[c]
2010	2	173	PT mainland	unknown[c]	unknown[c]	unknown[c]
2011	2	121	PT mainland	unknown[c]	unknown[c]	unknown[c]
2012 (Dec)/ 2013 (Jan)[a]	2	0	-	501	201	40.50%
2013 (second half)	3	428	Spain and PTmainland	unknown[c]	-	-
2014	4	664	Spain	unknown[c]	60	10.70%
2015	5	500[b]	Spain	unknown[c]	208	41.60%
2016 (Jan-April)	5	0	-	1500	130	8.67%

[a]-first outbreak of RHDV2 in the unit. [b]-approximated number. [c]-no records available

Table 1: Information on the number and origin of animals introduced into the unit between 2009 and 2015, estimated densities and registered casualties.

Citation: Carvalho CL, Rodeia J, Branco S, Monteiro M, Duarte EL, et al. (2016) Tracking the Origin of a Rabbit Haemorrhagic Virus 2 Outbreak in a Wild Rabbit Breeding Centre in Portugal; Epidemiological and Genetic Investigation. J Emerg Infect Dis 1: 114. doi:10.4172/2472-4998.1000114

The 2016 strains were compared with four strains from Barrancos obtained in previous years from dead wild rabbits collected in the same unit, two in January 2013 (KF442963 and KF442964) and other two in February 2014 (KM115675 and KM115676), as well as with two strains from Mértola (KM115712 and KM115713), a municipality located 81.22 km southwest from the unit, obtained in January 2014. The percent of nucleotide similarity among strains based on the *vp60* gene (Table 2) and the nucleotide differences and substitutions between viral populations showed concordant values (Table 3). Besides the six SNPs already mentioned above, the Barrancos-2016 strains differed from the Barrancos-2013 and from the Barrancos-2014 strains in 26 and 37 additional residues, accounting for a total of 32 and 43 mutations, respectively (Table 3). Interestingly, the Barrancos-2016 strains showed a slightly higher nucleotide similarity and lower nucleotide differences with the Barrancos-2013 strains than with Barrancos-2014 (Tables 2 and 3). The nucleotide variability found between the Barrancos-2013 and Barrancos-2014 strains (Table 3), involved 27 nucleotide variations. Regarding strains obtained in the same year in the Barrancos municipality, a similarity of 99.94% and 100% was observed. Among the oldest (2013 and 2014) and the 2016 strains, respectively (Table 2). No elations could be taken regarding the cumulative nucleotide variation of RHDV2 since the collection of the biological materials in each year occurred always within a short period of one month.

When comparing strains from the two municipalities, the Barrancos-2016 sequences (KX132812 and KX132813) showed 97.93% of similarity with the Mértola-2014 strains (Table 2), from which they differed in 31 nucleotides (Table 3).

The Bayesian tree constructed with the RHDV2 sequences presently available in public databases (May 2016) revealed that the Barrancos-2016 strains are more related with the Azorean strains (Figure 2) than with any other strain. The common branch for these two groups is supported by a high posterior probability of 0.94. While the position of the strains from Barrancos-2013 and Barrancos-2014 clearly differs from Barrancos-2016, their exact relationships with other strains from mainland South remains unresolved in the cladogram (Figure 2).

The ML tree (*results not shown*) presented the same topology of the Bayesian tree (Figure 2).

When all the strains obtained in the two neighbouring municipalities (Barrancos and Mértola) are compared, the Barrancos-2016 strains appear to be more closely related to the Mértola-2014 strains (KM115712 and KM115713) (Figure 2).

The phylogenetic network analysis (Figure 3) displayed a common putative intermediate ancestor for the RHDV2 Barrancos-2016 strains (KX132812 and KX132813) and those from Azores (KT000295, KT000303, KT000308, KT000311, KT000316-319, KT000322-25, KT000327, KT000329-330, KT000332-333, KT000336, KT000339, KT000341-343).

Also, the Barrancos-2013 strain KF442964 seems to represent a putative ancestor of KF442963 as well as for a number of strains that circulated in the South from 2013 to 2015 and Centre at least in 2014.

The analysis suggests a lineage for putative missing intermediates from which the Barrancos and Mértola strains may have arisen independently, with Mértola-2014 strains (KM115712 and KM115713) exhibiting an additional missing intermediate. Likewise, the network depicted an ancestral relation between the Barrancos-2014 strains (KM115675 and KM115676). Moreover, the two strains from Spain are more closely related with a strain from the North of Portugal mainland (KM979445), obtained from a wild rabbit in 2012 when the virus was first reported in the country [15].

Discussion and Conclusions

Immediately before release, the wild rabbits raised in the unit

			Barrancos						Mértola	
			2016		2014		2013		2014	
Geographic origin	Year of collection	Strain name	Barrancos1PT16	Barrancos2PT16	CBBarrancos14-1	CBBarrancos14-2	7-13_ Barrancos	10A-13_ Barrancos	CBMert 14-1	CBMert 14-2
		AC number	KX132812	KX132813	KM115675	KM115676	KF442963	KF442964	KM115712	KM115713
Barrancos	2016	**KX132812**	-	100	97.53	97.59	98.16	98.22	97.93	97.93
		KX132813		-	97.53	97.59	98.16	98.22	97.93	97.93
	2014	**KM115675**			-	99.94	98.45	98.51	97.99	97.99
		KM115676				-	98.51	98.57	98.05	98.05
	2013	**KF442963**					-	99.94	98.74	98.74
		KF442964						-	98.79	98.79
Mértola	2014	**KM115712**							-	100
		KM115713								-
Grey indicates the range of similarities (from lighter to darker: 97.53-97.99%; 98.45-98.79%; 99.94%;100%)										

Table 2: Percent similarity between nucleotide sequences of *vp60* gene from RHDV2 strains originated in Barrancos and Mértola municipalities.

Populations[a] compared			Total No. of mutations	No. of fixed differences	k	Average no. of nt differences between populations	Dxy	Da	Da(JC)±SD
Barrancos 2016	*Vs.*	Barrancos 2014	43	42	28,500	42,500	0,02443	0,02414	0,02454 ± 0,01521
Barrancos 2016	*Vs.*	Barrancos 2013	32	31	21,167	31,500	0,01810	0,01782	0,01804 ± 0,01123
Barrancos 2016	*Vs.*	Mértola 2014	37	35	24,333	36,000	0,02069	0,02011	0,02041 ± 0,01285
Barrancos 2014	*Vs.*	Barrancos 2013	27	25	17,667	26,000	0,01494	0,01437	0,01452 ± 0,00756
Barrancos 2014	*Vs.*	Mértola 2014	36	33	23,500	34,500	0,01983	0,01897	0,01923 ± 0,01006
Barrancos 2013	*Vs.*	Mértola 2014	23	20	14,833	21,500	0,01236	0,01149	0,01160 ± 0,00624

Table 3: Nucleotide differences and substitutions between the populations (defined as strains originated from the same municipality) considered in this study.

Citation: Carvalho CL, Rodeia J, Branco S, Monteiro M, Duarte EL, et al. (2016) Tracking the Origin of a Rabbit Haemorrhagic Virus 2 Outbreak in a Wild Rabbit Breeding Centre in Portugal; Epidemiological and Genetic Investigation. J Emerg Infect Dis 1: 114. doi:10.4172/2472-4998.1000114

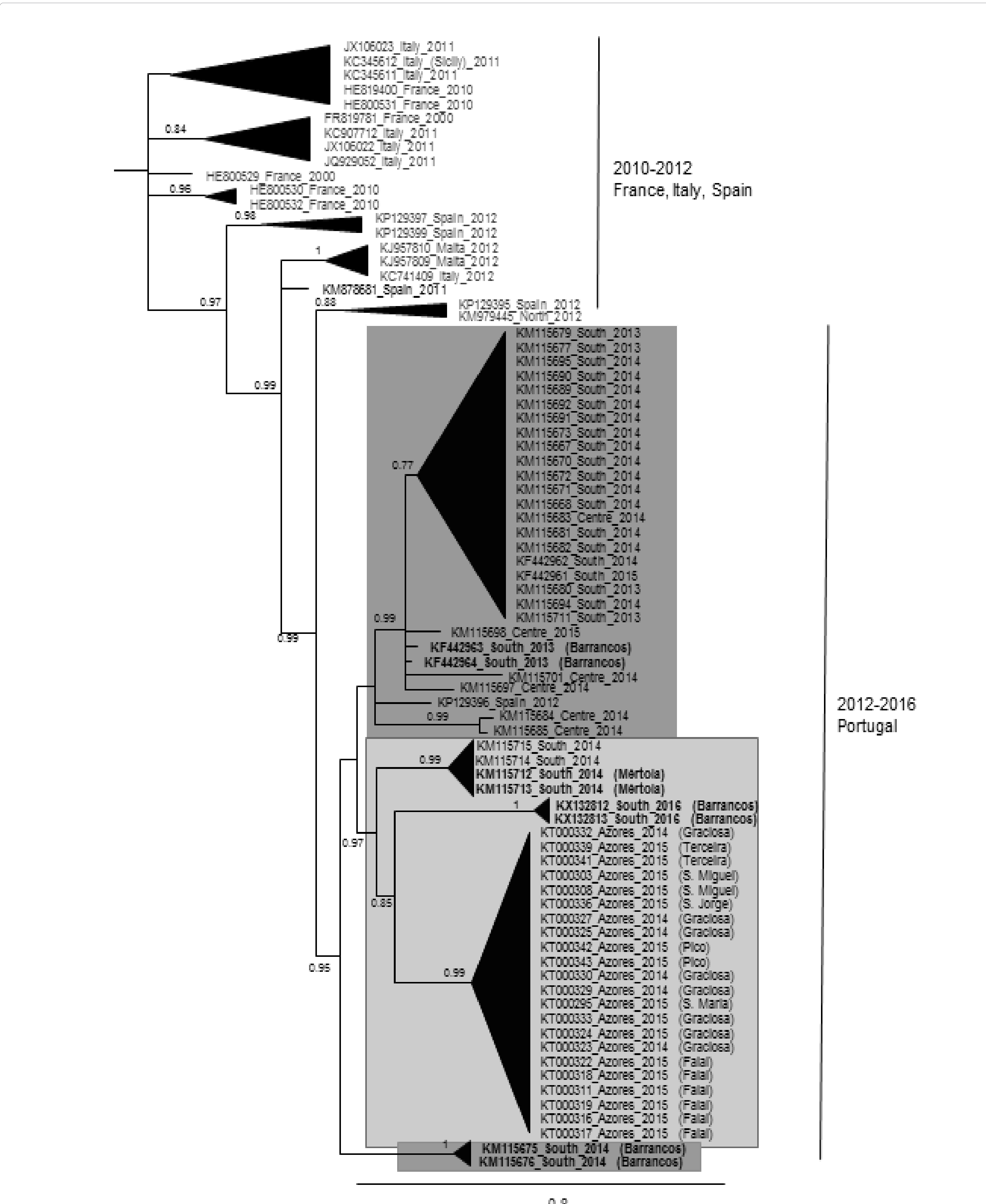


Figure 2: Bayesian analysis of the *vp60* complete nucleotide sequences from the Barrancos-2016 strains obtained during this study and other RHDV2 strains available in Genbank. A phylogenetic tree was obtained with a Bayesian inference of phylogeny throughout the MrBayes v3.1.2 software, using the GTR model (nst=6) with gamma-shaped rate variation with a proportion of invariable sites (rates=invgamma). The analysis was performed with ngen= 10^6, nchains=4 and samplefreq=10. The numbers included on each bootstrap represent the Bayesian posterior probability (PP). Only support PP values equal or greater than 0.70 are shown in the tree. Sequence KC345614R (classic RHDV, not displayed) was chosen as outgroup to root the tree.

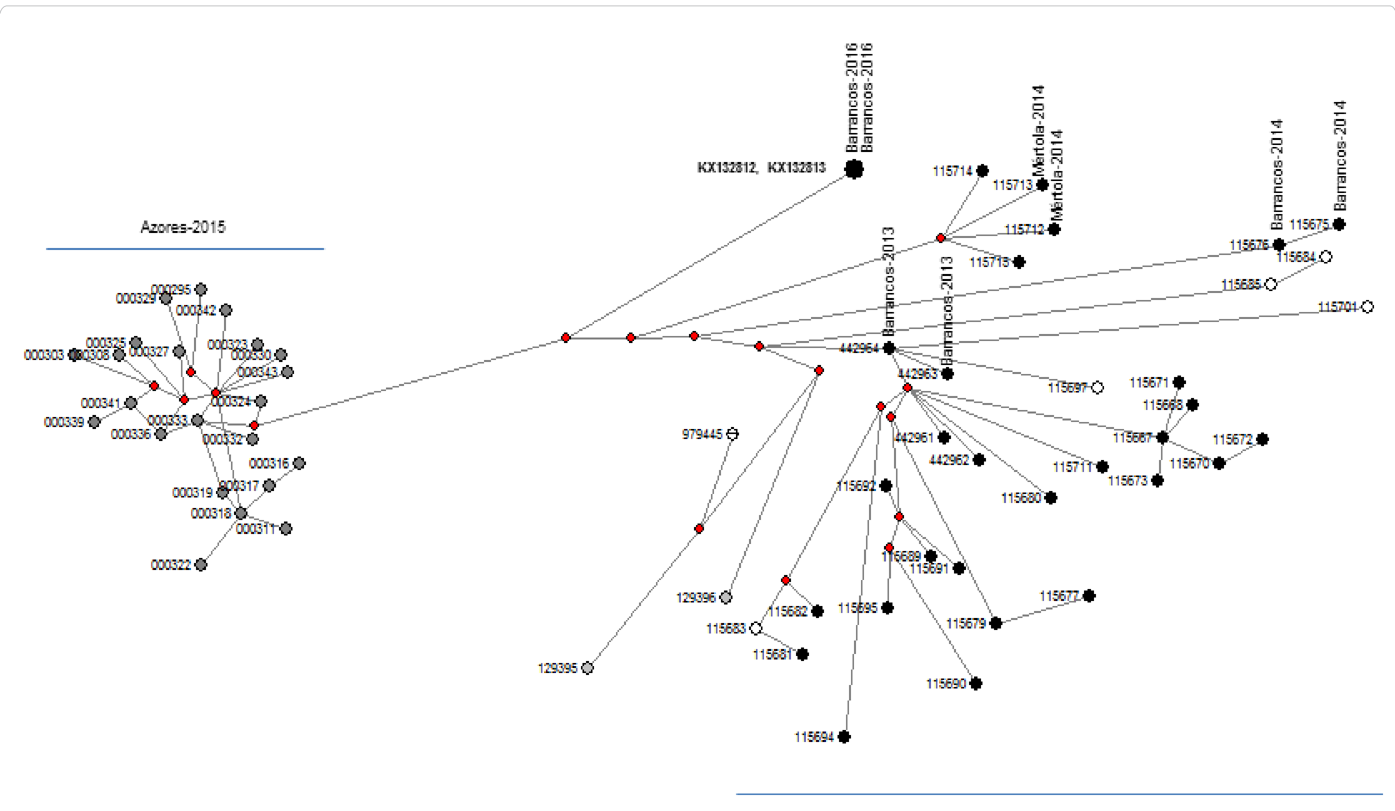


Figure 3: Median-joining phylogenetic network of RHDV2 viruses, constructed from the *vp60* sequence data. Each unique sequence genotype is represented by a circle sized relative to its frequency in the dataset. Sequences are coloured according to their geography origin: black circles denote strains from mainland South including Alentejo (strains from Barrancos and Mértola are indicated vertically); dark grey circles, strains from Azores; white circles from mainland Centre; vertical divided circles from mainland North; light grey circles from Spain. Missing intermediates are represented by red losangles.
This network includes the sequences highlighted by grey background in the phylogenetic tree. Accession numbers where edited to include only numbers, eg "KF442961" is shown as "442961": South (KF442961, KF442962, KM115680, KM115689, KM115692, KM115711, KM115695, KM115691, KM115682, KM115679, KM115667, KM115681, KM115690, KM115673, KM115671, KM115670, KM115668, KM115672, KM115694, KM115677, KM115712, KM115715), (KX132812, KX132813, KF442964, KF442965, KM115676, KM115675, KM115713, KM115714), Centre (KM115683, KM115701, KM115697, KM115684, KM115685), North (KM979445), Azores (KT000333, KT000324, KT000336, KT000332, KT000343, KT000342, KT000341, KT000330, KT000323, KT000319, KT000327, KT000325, KT000318, KT000295, KT000339, KT000329, KT000322, KT000317, KT000308, KT000316, KT000311, KT000303), Spain (KP129395, KP129396).

are vaccinated against myxoma virus and classical RHDV. However, RHDV2 vaccination, a growing practice in the domestic rabbit industry, is not practiced in this unit. Vaccination of captive wild rabbits against RHDV2 may provide temporary protection against infection and acquired immunity may increase survival of vaccinated rabbits if contact with the field strains occurs seven to 10 days after vaccination up to one year [32]. Vaccination is crucial for the establishment of herd immunity, although it can also be used as an effective post-exposure tool, in particular situations, for disease control according to the OIE [32]. The difficult feasibility of vaccine administration prior return to the wild, and the limited immunity induced by a single boost raises doubts about its contribution to a faster reestablishment of natural wild rabbit populations. Since RHDV2 vaccines are not freely available on the market, their use requires special licenses from the Veterinarian Authorities. Vaccination of wild captive rabbits against RHDV2 was exceptionally authorised by Portuguese National Authority for Animal Health (DGAV) in a few other wild rabbit breeding units.

For the revival of wild populations, the development of natural immunity against RHDV2 is crucial. Many factors will affect infection outcome at the population level, such as strain virulence [9,33], herd immunity due to previous infections [4,34] population density, season of the year [7,35], anthropogenic changes in the environment [4], landscape [9] and viral transmission between populations [4,9,36].

The geographic features of the unit, circumscribed by rivers, may offer a natural barrier to animal movement which is expected to slow down virus dissemination within the parks, similarly to what was described for RHDV [7,37], giving the virus more time to evolve [9]. This might explain the genetic diversity found between the strains from this unit obtained across this 4-years period. Also, the rapid transmission of a pathogen will affect the local spread of the disease reducing the number of susceptible individuals [4]. Hence, RHDV2 moderate virulence compared to RHDV, constitutes a selective advantage of this virus [9], as it was demonstrated that moderately virulent RHDV-related strains can invade wild rabbit populations with greater efficiency than highly virulent or non-pathogenic strains [9,33].

Interestingly, despite all the other parks in the unit were affected by RHDV2, there were no casualties recorded in P4. Since the virus first detection in the unit in 2012, animals from P4 seem to be passing unharmed. Although the factors behind the lower incidence of infection in P4 are not fully clarified, it is likely that its geographic isolation may have protected rabbits more efficiently from infection. However, the higher levels of humidity near the riverside probably also

favour mosquitoes, which are often implicated in disease transmission as mechanic vectors for RHDV [38], therefore puzzling this uneven occurrence of disease.

Also, an artificial boundary by electric fencing delimits each park adding up to the protection from predators given by several natural shelters located within the parks. Yet, the involving forestland provides suitable habitats for many free-ranging species including wild rabbits and small predators and scavengers such as badgers (*Meles meles*), mongooses (*Herpestes ichcneumon*), weasels (*Mustela nivalis*), foxes (*Vulpes vulpes*), stone martens (*Martes foina*), as well as birds of prey, often seen in the area, whose proximity with the parks may favour the direct or indirect contact with captive wild rabbits. Some predators and scavengers species can remove small animal carcasses from the site of death to other locations [39] and, concurring with that, a few specimens collected during this outbreak were damaged, probably due to scavenging. In fact, consumption of RHDV-victimized rabbits may be at the origin of the RHDV antibodies detected in red foxes and other predators and scavengers living in sympatry with affected rabbit populations [40-42]. These serological evidences pointed to the possibility of other species, apart from rabbits, being involved in the epidemiology and persistence of the disease [43].

Given the complex eco-epidemiology of RHDV2 depicted above, while efficient herd immunity is not achieved, serious difficulties may be posed to the establishment of self-sustainable wild rabbits populations in Baixo-Alentejo.

The phylogenetic analysis did not allow clarifying the origin of the infection since the Barrancos-2016 strains did not group with any other strain presently known, showing however to be more closely related with strains from Azores (supported by a PP of 0.85) (Figure 2). When looking to viruses originated in the same district, the Bayesian tree showed that Barrancos-2016 strains are more closely related with the Mértola-2014 strains than with other strains from Barrancos (pp of 0.99). The Barrancos-2013 strains clustered with strains from the South and Centre of Portugal mainland, while the two Barrancos-2014 strains grouped independently in a more ancestral branch, contradicting the temporal structure with regards to the position in the tree. However, these results were further clarified by the phylogenetic network analysis performed (Figure 3) where missing intermediates from which the Barrancos 2013, 2014 and 2016 strains may have evolved, present a chronological relative correct position. Based on the phylogenetic analyses, the Barrancos-2016 could represent an independent introduction of RHDV2 from unknown origin. However, it is worthy of mention that the unit is located in a preserved area were hunting activity is not allowed, therefore movement of hunters, which could be implicated in the virus dissemination from other locations, did not account for the occurrence of the outbreak.

Despite disease reports in the last five years suggest that RHDV2 may have circulated endemically in the region with epizootic occurrences, mortality was not observed in the immediate surrounding areas of the parks. Nonetheless, the possible involvement of the local wild rabbit population in the outbreak couldn't be proven or excluded, since the outside area was not systematically investigated prior the outbreak, a limitation of this study. The new virulent Barrancos-2016 strains could have evolved from previous circulating strains through cumulative mutations. The phylogenetic similarity with other strains isolated from Portugal, even though with no apparent geographical or epidemiological link, is more in favour of a common infection source rather than a less probable convergent evolution.

Finally, regarding rabbit translocations, the last introduction of animals in the park took place in January 2015, 14 months before the 2016 fatalities. It is unlikely that the virus was introduced from the Spain park supplier, where no records of RHDV2 infection were reported. Furthermore, during the at least 15 day quarantine carried out before translocation, no clinical signs of disease were observed.

The mortality rate estimations can contain some bias, a limitation inherent to the used methodology and related to the fact that in P2 and P3, in contrast with P1, rabbits have access to some seasonal natural food source, like acorn and grass, being also more vulnerable to predation, particularly by birds of prey. Nevertheless, the mortality rate estimated in 2016 was comparably lower than that observed in former years, namely in the outbreak between December 2012 and January 2013 (≈200 victimized animals) when the virus was first detected in the unit. At the time, the centre encompassed two parks with 200 and 300 animals, were a mortality rate of 42% and 39% (average 40.5%) was registered. This lower mortality rate compared with other reported outbreaks may be the reflex of a gradual RHDV2 immunity development in wild rabbit populations and its passive transmission to the offspring, a promising sign towards the establishment of host-virus equilibrium.

References

1. Dalton KP, Abrantes J, Lopes AM, Nicieza I, Alvarez AL, et al. (2015) Complete genome sequence of two rabbit hemorrhagic disease virus variant b isolates detected on the Iberian Peninsula. Arch Virol 160: 877-881.
2. Holmes EC (2004) The phylogeography of human viruses. Mol Ecol 13: 745-756.
3. Domingo E, Martínez-Salas E, Sobrino F, de la Torre JC, Portela A, et al. (1985) The quasispecies (extremely heterogeneous) nature of viral RNA genome populations: biological relevance--a review. Gene 40: 1-8.
4. Boots M, Hudson PJ, Sasaki A (2004) Large shifts in pathogen virulence relate to host population structure. Science 303: 842-844.
5. Harrison LH, Jolley KA, Shutt KA, Marsh JW, O'Leary M, et al. (2006) Maryland Emerging Infections Program. Antigenic shift and increased incidence of meningococcal disease. J Infect Dis 193-1266-1274.
6. Kerr PJ, Kitchen A, Holmes EC (2009) Origin and phylodynamics of rabbit hemorrhagic disease virus. J Virol 83: 12129-12138.
7. Alda F, Gaitero T, Suarez M, Merchan T, Rocha G, et al. (2010) Evolutionary history and molecular epidemiology of rabbit haemorrhagic disease virus in the Iberian Peninsula and Western Europe. BMC Evol Biol 10: 347.
8. Liu SJ, Xue HP, Pu BQ, Qian NH (1984) A new viral disease of rabbits. Animal Husbandry Vet Med 16: 253-255.
9. Le Gall-Recule G, Lavazza A, Marchandeau S, Bertagnoli S, Zwingelstein F, et al. (2013) Emergence of a new lagovirus related to Rabbit Haemorrhagic Disease Virus. Vet Res 44: 81.
10. Delibes-Mateos M, Delibes M, Ferreras P, Villafuerte R (2008) Key role of European rabbits in the conservation of the Western Mediterranean basin hotspot. Conserv Biol 22: 1106-1117.
11. Ferrer M, Negro JJ (2004) The Near Extinction of Two Large European Predators: Super Specialists Pay a Price. Conserv Biol 18: 344-349.
12. Rodríguez A, Delibes M (1992) Current range and status of the Iberian lynx Felis pardina Temminck, 1824 in Spain. Biol Conserv 61: 189-196.
13. IUCN (2003) IUCN Red List of Threatened Species.
14. Simon MA, Gil-Sanchez JM, Ruiz G, Garrote G, McCain EB, et al. (2012) Reverse of the decline of the endangered Iberian lynx. Conserv Biol 26: 731-736.
15. Abrantes J, Lopes AM, Dalton KP, Melo P, Correia JJ, et al. (2013) New variant of rabbit hemorrhagic disease virus, Portugal, 2012-2013. Emerg Infect Dis 19: 1900-1902.
16. Lopes AM, Correia J, Abrantes J, Melo P, Ramada M, et al. (2015) Is the new variant RHDV replacing genogroup 1 in Portuguese wild rabbit populations? Viruses 7: 27-36.

17. Duarte M, Henriques M, Barros SC, Fagulha T, Ramos F, et al. (2015) Detection of RHDV variant 2 in the Azores. Vet Rec 176: 130.

18. Delibes-Mateos M, Ferreira C, Carro F, Escudero MA, Gortazar C (2014) Ecosystem effects of variant rabbit hemorrhagic disease virus, Iberian Peninsula. Emerg Infect Dis 20: 2166-2168.

19. Delibes-Mateos M, Ferreras P, Villafuerte R (2007) Rabbit populations and game management: the situation after 15 years of rabbit haemorrhagic disease in central-southern Spain. Biodivers Conserv 17: 559-574.

20. Marchandeau S, Aubineau J, Berger F (2006) Abundance indices: reliability testing is crucial-a field case of wild rabbit Oryctolagus cuniculus. Wildlife Biol.

21. Norbury G (2001) Advances in New Zealand mammalogy 1990–2000: lagomorphs. J R Soc New Zeal.

22. Duarte MD, Carvalho CL, Barros SC, Henriques AM, Ramos F, et al. (2015) A real time Taqman RT-PCR for the detection of rabbit hemorrhagic disease virus 2 (RHDV2). J Virol Methods 219: 90-95.

23. Tham KM, Barnes SM, Hunter SN (1999) Polymerase chain reaction amplification and gene sequence analysis of a calicivirus from a feral rabbit. Virus Genes 18: 235-242.

24. Duarte MD, Barros SC, Henriques AM, Fagulha MT, Ramos F, et al. (2014) Development and validation of a real time PCR for the detection of myxoma virus based on the diploid gene M000.5L/R. J Virol Methods 196: 219-224.

25. Sievers F, Wilm A, Dineen D, Gibson TJ, Karplus K, et al. (2011) Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. Mol Syst Biol 7: 539.

26. Librado P, Rosas (2009) DNA Sp v.5 A software for comprehensive analysis of DNA polymorphism data. Bioinformatis 25: 1451-1452

27. Tavare S (1986) Some probabilistic and statistical problems in the anal- ysis of DNA sequences. In: Miura RM (Ed). Lectures on math- ematics in the life sciences. Volume 17. Providence (RI):American Mathematical Society. 57-86.

28. Maddison WP, Maddison DR (2009) Mesquite: a modular system for evolutionary analysis.

29. Huelsenbeck JP, Ronquist F, Nielsen R, Bollback JP (2001) Bayesian inference of phylogeny and its impact on evolutionary biology. Science 294: 2310-2314.

30. Ronquist F, Huelsenbeck JP (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19: 1572-1574.

31. Bandelt HJ, Forster P, Rohl A (1999) Median-joining networks for inferring intraspecific phylogenies. Mol Biol Evol 16: 37-48.

32. OIE Terrestrial manual (2016) Rabbit haemorrhagic disease.

33. Fouchet D, Le Pendu J, Guitton JS, Guiserix M, Marchandeau S, et al. (2009) Evolution of microparasites in spatially and genetically structured host populations: the example of RHDV infecting rabbits. J Theor Biol 257: 212-227.

34. White PJ, Norman RA, Hudson PJ (2002) Epidemiological consequences of a pathogen having both virulent and avirulent modes of transmission: the case of rabbit haemorrhagic disease virus. Epidemiol Infect 129: 665-677.

35. Cooke BD (2002) Rabbit haemorrhagic disease: field epidemiology and the management of wild rabbit populations. Rev Sci Tech 21: 347-358.

36. Calvete C (2006) Modeling the effect of population dynamics on the impact of rabbit hemorrhagic disease. Conserv Biol 20: 1232-1241.

37. Muller A, Freitas J, Silva E, Le Gall-Recule G, Zwingelstein F, et al. (2009) Evolution of rabbit haemorrhagic disease virus (RHDV) in the European rabbit (Oryctolagus cuniculus) from the Iberian Peninsula. Vet Microbiol 135: 368-373.

38. McColl KA, Merchant JC, Hardy J, Cooke BD, Robinson A, et al. (2002) Evidence for insect transmission of rabbit haemorrhagic disease virus. Epidemio Infect 129: 655-663.

39. Santos SM, Carvalho F, Mira A (2011) How long do the dead survive on the road? Carcass persistence probability and implications for road-kill monitoring surveys. PLoS One 6: e25383.

40. Frölich K, Klima F, Dedek J (1998) Antibodies against rabbit hemorrhagic disease virus in free-ranging red foxes from Germany. J Wildl Dis 34: 436-442.

41. Leighton FA, Artois M, Capucci L, Gavier-Widen D, Morisse JP (1995) Antibody response to rabbit viral hemorrhagic disease virus in red foxes (Vulpes vulpes) consuming livers of infected rabbits (Oryctolagus cuniculus). J Wildl Dis 31: 541-544.

42. Parkes JP, Heyward RP, Henning J, Motha MXJ (2004) Antibody responses to rabbit haemorrhagic disease virus in predators, scavengers, and hares in New Zealand during epidemics in sympatric rabbit populations. NZ Vet J 52: 85-89.

43. Merchan T, Rocha G, Alda F, Silva E, Thompson G, et al. (2011) Detection of rabbit haemorrhagic disease virus (RHDV) in nonspecific vertebrate hosts sympatric to the European wild rabbit (Oryctolagus cuniculus). Infect Genet Evol 11: 1469-1474.

Citation: Carvalho CL, Rodeia J, Branco S, Monteiro M, Duarte EL, et al. (2016) Tracking the Origin of a Rabbit Haemorrhagic Virus 2 Outbreak in a Wild Rabbit Breeding Centre in Portugal; Epidemiological and Genetic Investigation. J Emerg Infect Dis 1: 114. doi:10.4172/2472-4998.1000114

OMICS International: Publication Benefits & Features

Unique features:

- Increased global visibility of articles through worldwide distribution and indexing
- Showcasing recent research output in a timely and updated manner
- Special issues on the current trends of scientific research

Special features:

- 700+ Open Access Journals
- 50,000+ editorial team
- Rapid review process
- Quality and quick editorial, review and publication processing
- Indexing at major indexing services
- Sharing Option: Social Networking Enabled
- Authors, Reviewers and Editors rewarded with online Scientific Credits
- Better discount for your subsequent articles

Submit your manuscript at: http://www.omicsonline.org/submission/

Study 7

Where did the Berlengas - 2016 outbreak virus come from?

Detection of Rabbit Haemorrhagic Disease Virus 2 during the wild rabbit (*Oryctolagus cuniculus)* eradication from the Berlengas archipelago, Portugal

Fábio Abade dos Santos[1]*, Carina L. Carvalho[2]*, Nuno Oliveira[3], Jorge J. Correia[1], Ana M. Henriques[4], Maria Conceição Peleteiro[1], Miguel Fevereiro[4], Margarida D. Duarte[4]

*These authors contributed equally to this work.

[1] CIISA, Interdisciplinary Centre of Research in Animal Health, Faculdade de Medicina Veterinária (FMV), Universidade de Lisboa, Lisboa, Portugal; [2]Instituto de Ciências Agrárias e Ambientais Mediterrânicas (ICAAM), Universidade de Évora, Évora, Portugal; [3]Sociedade Portuguesa para o Estudo das Aves (SPEA), Portugal; [4]Instituto Nacional de Investigação Agrária e Veterinária (INIAV), Lisboa, Portugal;

Manuscript accepted for publication in *BMC Veterinary Research Journal*

1. Abstract

In the regular wildlife monitoring action carried out in the summer of the past few years, dead rabbits (*Oryctolagus cuniculus*) have been repeatedly found at the Berlenga Island. However, the origin of those deaths was never investigated.

The rabbits' population of the Berlenga Island, presently being eradicated, is a mixture of wild rabbits introduced in the island several centuries ago, with domestic rabbits brought into the island more recently.

In this study, 11 rabbit cadavers collected between April and May 2016 were investigated for the cause of death.

Tissue samples were screened for highly pathogenic agents, namely the haemorrhagic disease virus 2 (RHDV2), the closely related RHDV, and myxoma virus (MYXV). Five animals tested positive to RHDV2 and all were negative for the other viruses. For six RHDV2-negative specimens, emaciation and parasitism were considered the most probable cause of death. Lesions identified in the RHDV2-positive rabbits included non-suppurative diffuse hepatic necrosis and pulmonary lesions varying from congestion and oedema of the lungs to interstitial pneumonia. Sequencing analysis of the vp60 gene obtained from two specimens showed identical vp60 sequences. Comparison with other known RHDV2 strains from public databases through BLAST analysis revealed a closer similarity with strains from Alentejo collected during 2013. Maximum Likelihood and Bayesian phylogenetic analysis showed that the 2016 strains from the archipelago have a higher resemblance with a group of strains mostly collected in the South of Portugal between 2013 and 2014. These data suggest that RHDV2 may have been introduced on the Berlenga island a few years ago, having evolved separately from other strains due to insularity.

2. Keywords: Rabbit haemorrhagic disease virus, RHDV2, wild rabbit, *Oryctolagus cuniculus*, Berlengas, Berlenga Island, UNESCO

3. Introduction

The Berlengas (Figure 1) is a small archipelago located 5.5 nautical miles (about 10 km) west off Peniche, a fishing town in the Portuguese Atlantic coast. It encompasses the Berlenga Island (1,500 by 800 metres long with a surface area of 80 hectares), a group of small surrounding islets as well as two other more distant groups of islets (Estelas and Farilhões), being designated as Natural Reserve since 1981.

Berlenga is the only island that receives tourists, holding only a small group of houses used seasonally by local fishermen. During the 15th century, this island was a popular hunting royal province, probably due to the high population of European rabbits (*Oryctolagus cunniculus*), which exceptional abundance was reported already in 1465 (Amado et al, 2007).

Presently, Berlenga is the only Island from this archipelago where wild rabbits are present, with a population size estimated at 38 to 133 individuals, calculated through several transects performed over 2015 and 2016 (Figure 2) (Oliveira N, unpublished data). It is assumed that rabbits of unknown origin were brought onto this island for the first time during the 15th century (Amado et al., 2007). Lighthouse keepers are known to have taken domestic rabbits into the island for backyard farming, on different occasions during the last two centuries. Those animals eventually escaped and mixed together with the remaining population (Amado et al., 2007). Recent molecular data analysis has proved the presence of domestic genome on Berlenga Island's rabbits (Oliveira N, unpublished data). Over the last six centuries, rabbits have deeply changed the soil dynamics thereby speeding up erosion due to their digging behaviour (Amado et al., 2007). Also, the detrimental impact of *Rattus rattus* (Linneaus 1758) on the Berlengas ecosystem was recognized (Amado et al., 2007). Those reasons are leading a current process to eradicate rabbit and black rat populations from the Berlenga Island.

In the last couple of years, an abnormal mortality of rabbits has been observed on the island by marine biologists that are regular visitors (Oliveira N, personal communication). However, neither infectious pathogens nor toxicological agents were ever investigated to determine the origin of those deaths. Furthermore, to the best of our knowledge, no serological or pathogen surveys have been conducted in the past on the rabbit population of the island. The only available data originates from a study on the ecology of rabbits and black rats (*Rattus rattus*) ectoparasites (Pinto, 1995).

Among the pathogens that most severely affect rabbit, myxoma virus (MYXV) (Aragão, 1927)(Fenner and Ratcliffe, 1965)(Sanarelli, 1898) and rabbit haemorrhagic disease virus (RHDV) (Liu et al., 1984) assume a major relevance and their rate of transmission in the wild is affected by fluctuations in the wild rabbits' population density (Gonçalves et al., 2002)(Henning et al., 2005).

Myxoma virus (MYXV), a large dsDNA virus, is a member of the *Leporipoxvirus* genus of the family *Poxviridae*, subfamily *Chordopoxvirinae*, (Kerr et al., 2015). It appears to be passively transmitted to European and American rabbits (*Sylvilagus brasiliensis* and S. *bachmani*) as well as to hares (*Lepus europaeus*) through the biting of insects that act as mechanical vectors, once the virus adheres to

their mouthparts (Kerr et al., 2015) (Kerr, 2012) (Brugman et al., 2015). In its natural host (*S. brasiliensis*) and in hares, MYXV rarely causes disease (Kerr et al., 2015), but European domestic and wild rabbits may develop a rapid systemic infection causing death in few days (Kerr et al., 2015).

Rabbit haemorrhagic disease (RHD) is a highly contagious infectious disease of the European wild and domestic rabbits, caused by a virus of the *Lagovirus* genus, family *Caliciviridae*. RHD is characterised by high morbidity and mortality: 70–90% for RHDV/RHDVa and 5–70% for RHDV2 (OIE Terrestrial Manual, 2016). The disease was first identified in 1984 in China (Liu et al., 1984). In Europe, RHD was first diagnosed in Italy in 1986 (Cancellotti and Renzi, 1991), soon becoming endemic in several countries (Abrantes et al., 2012). In the Iberian Peninsula, from where European rabbits originated and where they are key species of the ecosystem (Delibes-Mateos et al., 2008), the first outbreaks occurred in 1988 in Spain (Argüello Villares et al., 1988) and in the following year in Portugal (Anonymous, 1989)(Abrantes et al., 2012), causing severe reduction of the wild rabbit populations (Villafuerte et al, 1995)(Abrantes et al., 2012) and considerable economic losses in the rabbit industry (Argüello Villares et al., 1988). Despite the low level of genetic variation found, the molecular characterization of RHDV strains allowed the distinction of six well-defined phylogenetic genogroups (G1 to G6) (Nowotny et al., 1997)(Le Gall et al., 1998).

In 2010, a new virus designated RHDV2 emerged with a distinct genetic and antigenic profile (Le Gall-Reculé et al., 2011). It was first identified in France in 2010 (Le Gall-Reculé et al., 2011), and rapidly spread to several European countries including Italy (Le Gall-Reculé et al., 2013), Spain (Dalton et al., 2012), Portugal (Abrantes et al., 2013), the United Kingdom (Westcott et al., 2014) and Scotland (Baily et al., 2014). Outside the European continent, RHDV2 was first detected between late 2014 and early 2015, in the Azores archipelago (Duarte et al., 2015a). More recently, RHDV2 was also reported in Australia (Hall et al., 2015), Finland (http://www.oie.int/wahis_2/public/wahid.php/Countryinformation/Countryreports) and North of Africa (Martin-Alonso et al., 2016).

RHDV2 differs from RHDV in the clinical characteristics of the induced disease in terms of duration, mortality rates and the more frequent occurrence of subacute/ chronic forms (Le Gall-Reculé et al., 2013). Furthermore, RHDV2 also affects young rabbits with less than two months old, often before they leave the burrows, as well as RHDV vaccinated rabbits that, although protected against classical strains, are susceptible to RHDV2 infection (Le Gall-Reculé et al., 2011)(Dalton et al., 2012)(Le Gall-Reculé et al., 2013). In addition, RHDV2 is also able to infect hosts other than rabbits

as the virus was detected in cape hares (*Lepus capensis*) (Puggioni et al., 2013) as well as Italian hares (*Lepus corsicanus*) (Camarda et al., 2014).

In Portugal, since RHDV2 cases were found (Abrantes et al., 2013), the former circulating classical RHDV genogroups, mostly G1-related strains in mainland (Muller et al., 2009)(Alda et al., 2010) and G5 in Azores (Duarte et al., 2014a), were no longer identified, suggesting that RHDV2 replaced the classical RHDV strains probably due to a selective advantage of this new virus by overcoming the existing immunity to older strains (Lopes et al., 2015).

In this study, we aimed to investigate the cause of death of wild rabbits on the Berlenga Island, occurring in the spring of 2016.

4. Material and Methods

4.1 Sample collection

The rabbits used in this study were collected on the Berlenga Island, Portugal, between April and May 2016, within the scope of the LIFE Berlengas project ("Conserving threatened habitats and species in Berlengas SPA through sustainable management", LIFE13 NAT/PT/000458).

Eleven cadavers, consisting of seven females (64%) and four males (26%), were selected from a larger group, based upon their good preservation status. All collected specimens were weighed. Age was estimated upon tarsus and skull length (occipitonasal length), measured with a micrometre. Skull length was defined as the highest distance from the cranial extremity of the premaxillae (excluding the incisors) to the rear of the occipital crest. This age estimation was determined according to the equation first used by Southern (1940) (Southern, 1940) and revised by Dunnet (1956) (Dunnet and Dunnet, 1956). Prior to freezing, liver samples were collected. All cadavers were then frozen at -20°C until necropsy.

4.2 Virological examination

At the National Reference Laboratory (INIAV), rabbit pathogens associated with high mortality rates, namely RHDV, RHDV2 and MYXV, were investigated. Liver samples from the 11 specimens were homogenised with phosphate buffered saline (PBS) and clarified at 3,000 g for 5 min. DNA and RNA were extracted from 200 µl of the clarified supernatant, corresponding to approximately 50 mg of tissue, in a BioSprint 96 nucleic acid extractor (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Samples were tested for RHDV2 by a specific RT-qPCR (Duarte et al., 2015b). Screening for RHDV (Tham et al., 1999) was performed by sequencing of the amplicons

obtained with primers RC9F and RC10R (Tham et al., 1999). Conventional RT-PCR and RT-qPCR were performed with the One Step RT-PCR kit (Qiagen, Hilden, Germany).

The presence of myxoma virus was investigated by qPCR (Duarte et al., 2014b), using the FastStart TaqMan Probe Master Kit (Roche, Roche Diagnostics GmbH, Manheim, Germany).

For the real-time PCR systems described, undetectable Cq or Cq values >40 were considered negative.

4.3 Nucleotide sequencing analysis

Amplification of the complete vp60 sequences of RHDV2 strains was accomplished with two pairs of primers, 27F (5'-CCATGCCAGACTTGCGTCCC-3') and 986R (5'-AACCATCTGGAGCAATTTGGG-3'), 717F (5'-CGCAGATCTCCTCACAACCC-3') (Duarte et al., 2015c), and RC10R (Tham et al., 1999) enabling the obtainment of two overlapping fragments. Both 717F and 986R are specific for RHDV2. The One Step (Qiagen, Hilden, Germany) kit was used, under the manufacturers' recommendations. Sequencing was accomplished using the BigDye™ Terminator cycle sequencing kit (Applied Biosystems, Foster City, CA, USA).

The complete vp60 nucleotide sequences of two RHDV2 strains (GenBank accession numbers KY247124 and KY247125) were determined on an automated 3130 Genetic Analyzer system (Applied Biosystems, Foster City, CA, USA).

4.4 Phylogenetic analysis

The phylogenetic relationships between two RHDV2 vp60 sequences from the Berlenga Island with other RHDV2 sequences originated in Portugal mainland, Azores and worldwide, were investigated. Sequence KC345614 (from a classical RHDV strain belonging to genogroup G5) was chosen as outgroup to root the trees.

Multiple alignments of the nucleotide sequences were generated by CLUSTAL Omega (Sievers et al., 2011).

Phylogenetic inference was performed by Maximum Likelihood (ML) and Bayesian methods. For ML analysis, the appropriated substitution model was determined resourcing to R software (R Development Core Team, 2011). The GTR model (Tavaré et al., 1986) with gamma-distributed rate variation across sites (GTR+G) showed the lowest BIC and AICc values and was subsequently used to infer phylogenetic relationships. Robustness of the tree nodes was assessed by bootstrapping 1000 times.

For the Bayesian analysis, the CLUSTAL Omega results were converted to the NEXUS format using Mesquite software (Madison and Madison, 2009). The phylogenetic tree was obtained with a Bayesian inference of phylogeny throughout the MrBayes version 3.1.2 software that uses the Markov chain Monte Carlo simulation technique to approximate the posterior probabilities (pp) of trees (Huelsenbeck et al., 2001)(Ronquist and Huelsenbeck, 2003). MrBayes analysis was performed using the GTR model (nst=6) with gamma-shaped rate variation with a proportion of invariable sites (rates=invgamma). The analysis was run for 106 generations (ngen=106) with four chains of temperature (nchains=4), and each chain was sampled every 10th generations (samplefreq=10).
The graphical representation and edition of the phylogenetic trees were performed with FigTree v1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/).

4.5 Necropsy and histopathological examination

Necropsy and histopathological examination was carried out the Pathology Laboratory of the Faculty of Veterinary Medicine of the University of Lisbon.
Organs collected from necropsy (liver and lungs) were submitted to fixation in 10% buffered formalin and processed for routine histopathological analysis. Sections were stained with H&E and Pearls Blue and microphotographs were obtained with a DP23 Olympus digital camera.

5. Results

5.1. Morphometric data

Weight of the 11 selected rabbits varied between 330 and 600 g (10g sensitive scale). Mean tarsus and skull length was 44mm and 75mm, respectively corresponding to an estimated age of 43 to 83 days. All RHDV2-positive rabbits were less than six months old.

5.2 Virological examination

Six liver samples were negative to RHDV2 and RHDV. The other five tested positively to RHDV2 by RT-qPCR (Duarte et al., 2015b). None of the eleven rabbits showed cutaneous myxomas or were positive to myxoma virus by qPCR.
Sequencing of the complete vp60 gene obtained from two specimens confirmed the presence of RHDV2 circulating in the island. These vp60 sequences (KY247124 and KY247125) are identical (100% similarity).

5.3 Phylogenetic analysis

Blast analysis of the Berlengas' vp60 sequence showed higher similarity (99%) with strains from the South of Portugal mainland, in particularly with strains from Barrancos obtained in 2013.

ML (Figure 3) and Bayesian (not shown) trees were consistent, showing that the strains from the Berlenga Island are most closely related (Bootstrap value of 75) with a major group of strains originated mostly in the South between 2013 and 2014. No clear resemblance was noticed with any strain collected more recently.

5.4 Necropsy and histopathological examination

The six RHDV2-negative rabbits, all aged less than six months, showed severe emaciation with reduced fat stores and muscle mass. The hair coat was in poor condition (rude and dull) and faecal material was adherent to the perineum. At necropsy, macroscopic lesions were neither observed in the liver nor in any of the other organs except for the small intestines that were distended, with inflammation and oedema of the jejunum and ileum. No bleedings or mucosal ulcerations were however found. In the fecal smear of the jejunum and ileum contents *Eimeria* spp. oocysts were observed (results not shown).

Four of the five RHDV2-positive rabbits showed low body condition (score 1 in the 1 to 5 in body condition scoring for rabbits, according to PFMA, 2012). In all these specimens the noses were soiled by bloody discharge, and in three rabbits fluid faecal material in the perianal region was present. Organ preservation was considered good enough to assure lesions identification. Changes were mostly restricted to the respiratory apparatus and liver. Respiratory lesions, present in all rabbits, included congestion of the trachea, which contained serohaemorrhagic fluid, and congestion of the lungs. No signs of overt haemorrhage were detected. In all cases, there was discoloration and diminished consistency of the liver. Three rabbits, the same with soiled perianal region, showed distended caeca and had unsolidified faecal material in the colon. The fresh faecal smear analysis of the jejunum and ileum revealed the presence of *Eimeria* spp. oocysts.

Histology analysis of the liver showed diffuse hepatocyte coagulation necrosis with no pattern distribution, supporting a diagnosis of diffuse non-suppurative acute hepatic necrosis. Most hepatocytes were karyolytic and only very few displayed karyorrhexis or pyknosis (Figure 4 A and B). In one case, the necrotic hepatic cells revealed vacuolated profile. Iron pigment deposition in Kuppfer cells was regularly seen. Lung lesions varied from congestion and alveolar oedema (n=2) to inflammatory infiltrates in the alveolar septa by mononuclear cells with atelectasis and

inflammatory cells in the alveolar lumen, consistent with interstitial pneumonia (n=3) (Figure 5A and 5B).

6. Discussion and Conclusions

Notwithstanding rabbit mortality at the Berlenga Island had not been investigated in previous years, our study clearly shows that RHDV2 was circulating in the island, at least since May 2016, and caused the death of five of the eleven rabbits investigated that died during the last spring. It is known that limited food availability and predation by dogs are also a cause for rabbit mortality in the island. Also, given the scarcity of fresh water in the island, the rabbit population of the island is highly dependent on the rainfall regime, with the mortality rate increasing during the dry months (Vicente, 1989). Despite no detailed parasitological examination was carried out during this study, the five RHDV2-negative young found in April, presented signs of intestinal coccidiosis along with emaciation that could have contributed to their deaths. Also, apart from those findings, no lesions were found in the liver and lungs that could suggest haemorrhagic disease. The impact of coccidiosis, caused by *Eimeria* spp., in the European rabbit populations of the Iberian Peninsula was recently evaluated in two ecological regions of Spain, estimating the coccidian prevalence by the mean oocyst excretion levels detected in faeces. Although in both areas the oocyst per gram of faeces was generally low, six *Eimeria* species were identified (Silva et al., 2015). Among those (*E. coecicola*, *E. perforans*, *E media*, *E. magna*, *E. irresidua* and *E. flavescens*), *E. flavescens* is considered highly pathogenic for rabbits (Licois, 2004). Curiously, the abundance and loads of Coccidia and nematodes in reintroduced rabbit populations in Spain showed no clear pattern with rabbit haemorrhagic disease prevalence (Bertó-Moran et al., 2013).Nonetheless, the impact of coccidea infection on morbidity and mortality of wild rabbit populations of the Berlenga Island was never investigated.

Myxoma virus-DNA was not detected in any of the 11 rabbits tested. Furthermore, none of these animals exhibited skin lesions, oedema or signs of conjunctivitis suggestive of nodular myxomatosis disease. Also, the qPCR developed by Duarte et al. (Duarte et al., 2014b) has allowed to detect MYXV-DNA in several organs of wild rabbits (results not published, Duarbete et al.), which increases the confidence on the MYXV-PCR negative results obtained in the liver samples during this study.

RHDV2 infection was the cause of death of five rabbits from Berlenga Island collected in May 2016. However, given the reduced size of our sample, the positivity percentage of 45.5% (5/11 animals) is, most likely, merely indicative of the true mortality induced during the outbreak.

The characterization of RHDV2 induced lesions is still limited compared with that regarding RHDV. Post-mortem examination of RHDV2-infected rabbits revealed macroscopic lesions consistent with haemorrhages in several organs including heart, trachea, thymus, lungs, liver, kidneys, and gut, as well as jaundice (Dalton et al., 2012)(Duarte et al., 2015c). The liver appearance was described as soft and pale (Duarte et al., 2015c). Histopathologic descriptions refer to haemorrhagic pneumonia and tracheitis, congestion of the liver and diffuse necrotizing hepatitis. Areas of focal necrosis were also described in the intestinal villi in the small intestine (Dalton et al., 2012).

In the present report, lung lesions varied from simple congestion and alveolar oedema, indicating an acute evolution, to interstitial pneumonia suggesting a longer disease process. Haemorrhagic lesions were neither identified in the lungs nor in other organs, although rabbits consistently showed soiled noses, probably by blood tinged oedematous lung fluid. Interstitial pneumonia observed in three out of five rabbits have not been reported before in the post-mortem examination of RHDV or RHDV2 infected rabbits (Marcato et al., 1991)(McIntosh et al., 2007)(Duarte et al., 2015c)(Lopes et al., 2015) It is possible that pneumonia could have eventually be due to a longer disease evolution or to bacterial infections occurring prior to the contact with the RHDV2. Inflammatory infiltrates by mononuclear cells are quite compatible with viral infection (figure 5B).

In fact, the diffuse coagulation necrosis registered in the liver of all rabbits is only compatible with an acute or hyperacute course the disease possibly favoured by the low body condition of the rabbits. The type of necrosis with most of the cells karyolitic and without a defined pattern distribution is more in agreement with the lesions described in European Brown Hare Syndrome (EBHS) (Marcato et al., 1991), although the reports of RHDV and RDHV2 infected rabbits consistently refer to severe necrotic liver lesions (Marcato et al., 1991)(McIntosh et al., 2007)(Duarte et al., 2015c)(Lopes et al., 2015).

In summary, death of the infected rabbits analysed in this study must have been due to acute hepatic failure with lung congestion and oedema occurring closer to death. The relevance of the interstitial pneumonia remains unclarified as no other infectious agents were investigated apart from the aforementioned leporid-specific viruses. The fact that the cadavers were not fresh limited the confidence and significance in any bacterial identification.

The phylogenetic results revealed that the 2016 strains from the Berlenga Island formed an independent cluster in closer proximity with a group of sequences obtained between 2013 and 2014, mainly in the South of Portugal mainland. The higher resemblance with a group of older strains may suggest that RHDV2 was not introduced recently in the Island. Instead, and with accordance with the mortalities observed in the last years, RHDV2 introduction may have taken place a few years ago, circulating since then in the island. Because no intermediate strains between the Barrancos 2013 and Berlengas 2016 strains were found until the moment (results not shown), this possibility remains only hypothetical. Yet, the island geographic location may well have provided the necessary isolation for the Berlenga Island strains to evolve apart from other haplotypes that have been characterized in the latest years in Portugal mainland (Abrantes et al., 2013) (Carvalho et al., 2016) and in Azores (Duarte et al., 2015c)(Almeida et al., 2015). Due to the advanced stage of rabbit eradication at the time samples were collected, no serologic study could be carried out which may have allowed to clarify if RHDV2 was introduced on the island prior the 2016 outbreak.

Given the island eco-geographic particularities, RHDV2 introduction may have occurred by several routes. Beside human means and mechanical action of arthropod vectors (Asgari et al., 1998)(Cooke, 2002)(McColl et al., 2002), black rat may also have accounted for the viral introduction. Native from the Indian peninsula (Musser and Carleton 2005), the black rat is worldwide distributed (Brouat et al., 2014) and is the only rodent species found on the island (Amado et al., 2007). Although never demonstrated, similarly to *Apodemus silvaticus* and *Mus spretus* (Merchán et al., 2011), black rats from RHDV2 infected areas may have carried the virus to the rabbit population of Berlengas after being inadvertently transported by the boats that arrive at the island. Other possibilities concern resident birds of prey such as the Common Kestrel *(Falco tinnunculus*) and the Peregrine Falcon (*Falco peregrinus*) or vagrant birds as the Common Buzzard (*Buteo buteo*), that may also have carried contaminated leftovers from RHDV2 infected rabbits from Portugal mainland. Furthermore, Berlenga Island holds a large population of Yellow-legged Gulls (*Larus michahellis*), estimated near 13,150 individuals (Meirinho et al, 2014), which daily feed on waste treatment plants, farms and aviaries on the surrounding grounds of the Peniche municipality (located in mainland and which also includes the Berlengas archipelago) (Ceia et al., 2014). No reliable data could be gathered regarding the occurrence of haemorrhagic disease in domestic rabbits in Peniche. However, abnormal mortality rates have been observed in wild rabbits from local game reserves, Atouguia da Baleia, Ferrel and Serra d'el-Rei, during the last years.

RHDV2 infections have been demonstrated in most of the national territory since its introduction in 2012, drastically reducing the wild rabbit population in particular areas (Abrantes et al., 2013)(Almeida et al., 2015)(Duarte et al., 2015c)(Lopes et al., 2015). Therefore, it is highly likely that the three Peniche neighbouring parishes were the game reserves are sited may have also been affected. The Berlenga Island location near the coast, support the hypothesis that the virus strains found in the island may have been introduced from the nearby RHDV2 affected geographical area. Direct contact with rabbit contaminated materials is therefore likely to have occurred since Yellow-legged Gulls are commonly seen transporting dead corpses of several animals into the island including parts of rabbits (Oliveira N., personal communication).

The exceptional terrestrial insular ecosystem of Berlengas encompasses unique characteristics (Catry et al., 2010). The archipelago provides nesting conditions for seabird species from the families *Procellariidae, Hydrobatidae*, *Phalacrocoracidae*, and *Laridae*, such as the Cory's Shearwater (*Calonectris borealis*), the Band-rumped storm-petrel (*Hydrobates castro*) only present in Farilhões, the European Shag (*Phalacrocorax aristotelis*), the Yellow-legged Gull and the Lesser Black-backed Gull (*Larus fuscus*) (Catry et al., 2010). Cory's Shearwater, Band-rumped Storm-petrel and European Shag are classified as vulnerable by the Portuguese RedList Book (Cabral et al., 2005). The role of rabbits on deviation of the original ecology of the Berlenga Island is controversial. Besides the effect of rabbits, it is known that the decline of the unique endemic vegetation such as the *Armeria berlengensi*, *Herniaria berlengiana*, *Pulicaria microcephala* as well as the *Lobularia maritime*, and *Frankenia laevis*, is also a consequence of the acid excrements of the overpopulated Yellow-legged Gull and competition by Ice plant (*Carpobrotus edulis*), an invasive plant native to South Africa brought into the island in the 1980's and which threatens its natural vegetation biodiversity (Gomes et al., 2004).

The current conservation policy of the Berlengas archipelago biological heritage, classified as of high interest, is due to its protected habitats and vulnerable surrounding marine ecosystem, one of the richest Portuguese seawaters for which it was considered Nature Reserve by UNESCO since 2010. Aiming to guarantee the survival of several endangered species, a national plan to eradicate the rabbit and black rat population from Berlenga is in course. If successful, this study may have been the last opportunity to trace the RHDV2 passage through the Berlengas archipelago.

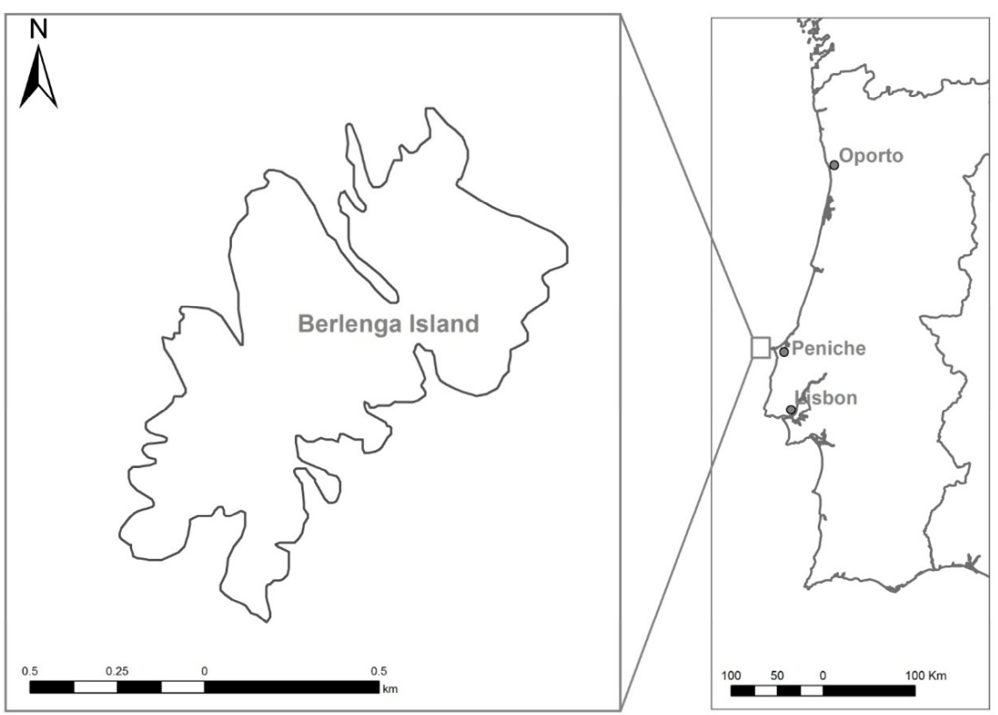


Figure 1. Berlengas Island, the major island of the Berlengas' archipelago, located in the Portuguese maritime coast, and Peniche municipality (Portugal mainland) are marked on the map.

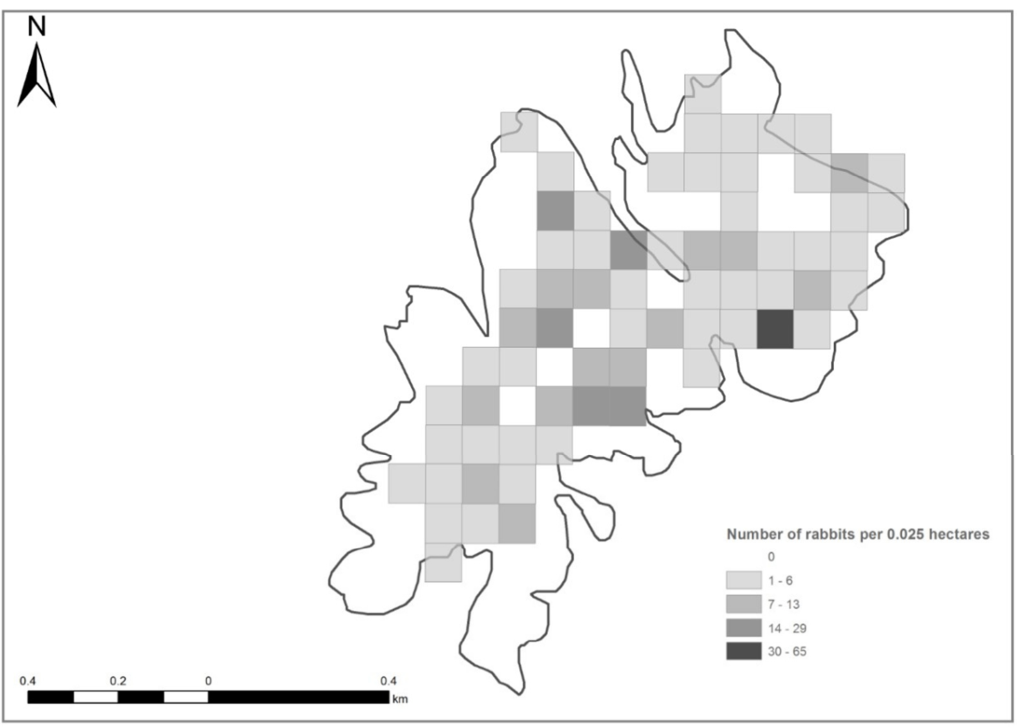


Figure 2. Density of the wild rabbit population in the Berlengas Natural Reserve calculated through several transects performed over 2015 and 2016.

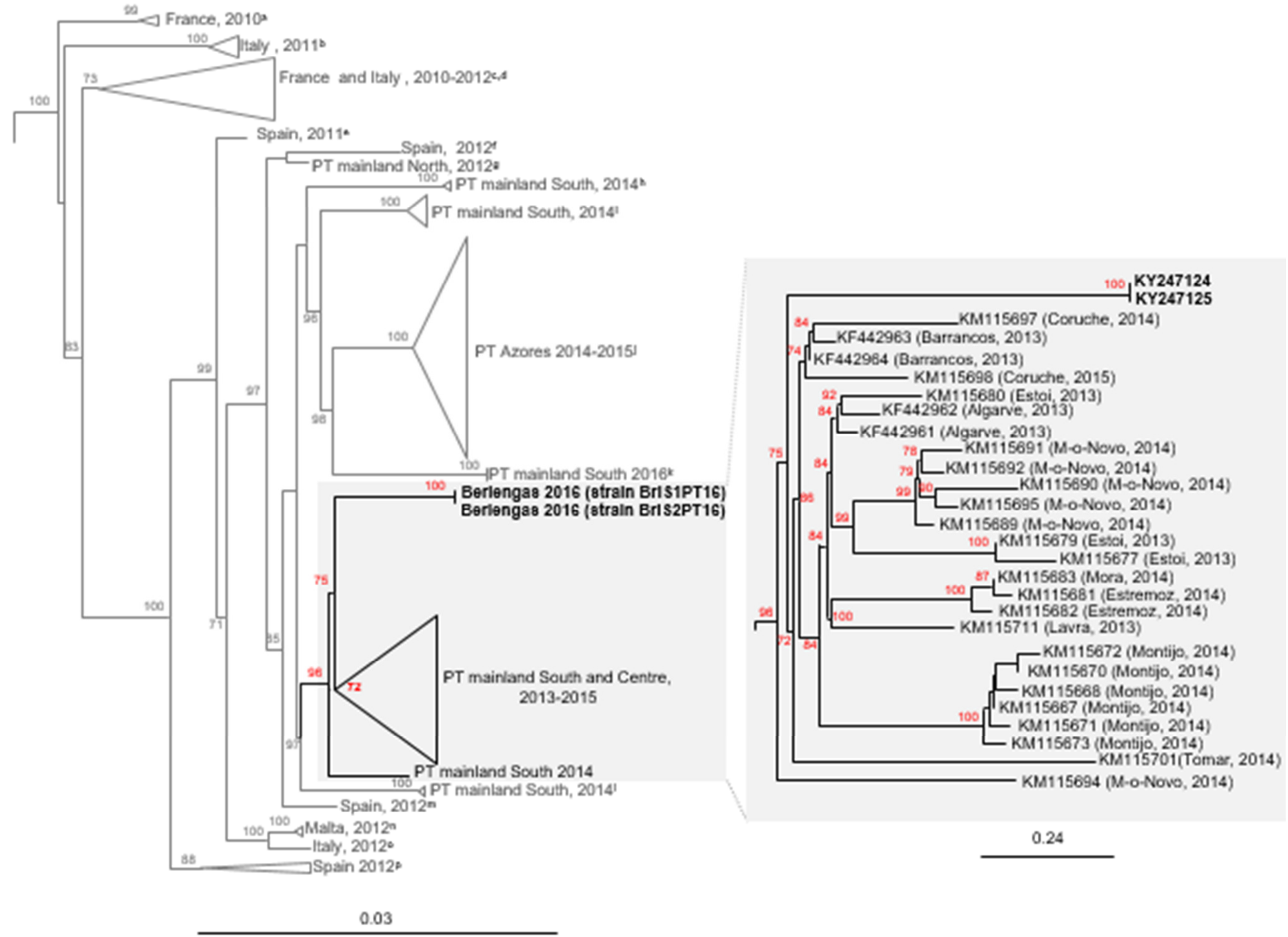
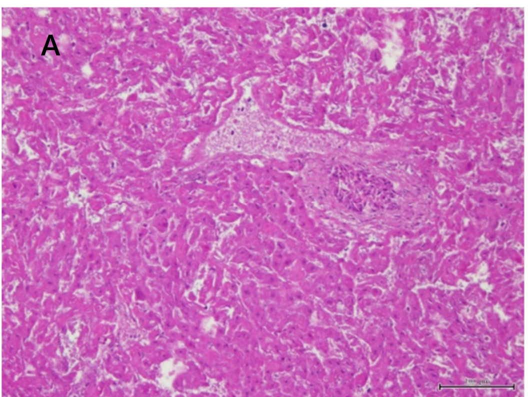


Figure 3. Maximum Likelihood (ML) phylogenetic tree of the RHDV2 vp60 complete nucleotide sequences from the Berlenga island (2016) and others originated in Portugal mainland, Azores, Spain, France, Italy and Malta, available in Genbank. Bootstrap values (BS) are shown next to the nodes if equal or greater than 70. Sequence KC345614 (not displayed) was chosen as outgroup to root the tree.

In the left side tree, the major groups are collapsed to facilitate visualization. a-HE800531, HE819400; b-JX106023, KC345611-12; c-HE800529, HE800530, HE800532 and FR819781; d-JQ929052, KC907712 and JX106022; e-KM87868; f-KP129395; g-KM979445; h-KM115675-76; i-KM115712-13; j-KT000295, KT000303, KT000308, KT000311, KT000316-319, KT000322-325 KT000327, KT000329-330, KT000332-333, KT000336, KT000339 and KT000341-343; k-KX132812 and KX132813; l-KM115684-5; m-KP129396; n-KJ957809 and KJ957810; o-KC741409; p-KP129397 and KP129399. “M-o-Novo” refers to the municipality of Montemor-o-Novo.

A close-up of the RHDV2 strains more closely related with the strains from the Berlenga Island is shown in the right side of the figure.



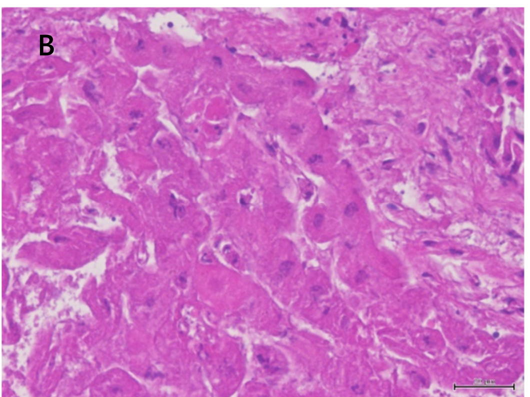
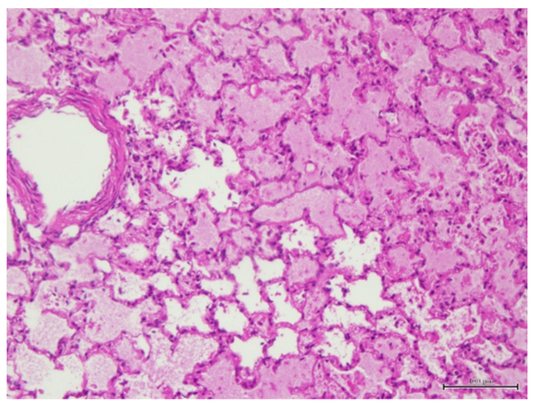


Figure 4. Liver of rabbit positive to RHDV2. A - Diffuse hepatocyte coagulation necrosis with no pattern distribution. Most hepatocytes are karyolytic and only very few display karyorrhexis or pyknosis. B – Detail of A showing necrotic hepatic cells displaying karyorrhexis and pyknosis (H&E).



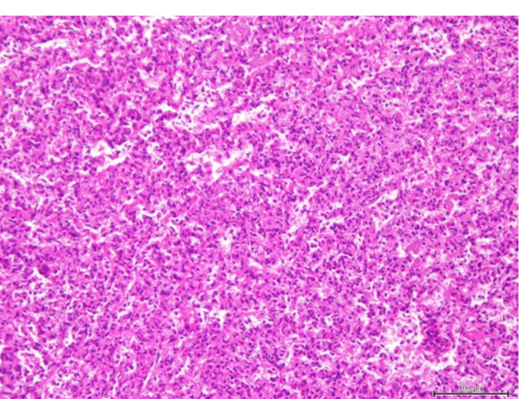


Figure 5. Lung of rabbits positive to RHDV2. A – Alveolar congestion and oedema. B-Interstitial pneumonia with inflammatory infiltrates in the alveolar septa by mononuclear cells, which are also present in the alveolar lumen (H&E).

7. Acknowledgements

We are thankful to João Teixeira, Fátima Cordeiro, Sandra Carvalho and Rosário Luís for excellent technical assistance. We also present our acknowledgments to Berlengas Natural Reserve guards (Paulo Crisóstomo, Eduardo Mourato, Filipe Correia, Tiago Menino and António Figueiredo), all volunteers and SPEA staff (namely Isabel Fagundes and Pedro Luís Geraldes) who supported the entire field work. Thanks to the National Maritime Authority and Peniche Captaincy for the transport to and from the Berlenga Island. Finally, thank you to the Instituto de Conservação da Natureza e das Florestas, namely to Maria de Jesus Fernandes and Maria de Lurdes Morais for their full support to this study. Necropsies and histopathologic analysis were supported by the Interdisciplinary Research Centre on Animal Health, Faculty of Veterinary Medicine, University of Lisbon, (CIISA, FMV, UL) (Portugal).

Molecular analysis was partially funded by the Fundação para a Ciência e Tecnologia (FCT) (grant SFRH/79225/2011) and and Instituto de Ciências Agrárias e Ambientais Mediterrânicas (ICAAM) (Project UID/AGR/00115/2013).

Field work was supported by the LIFE + Berlengas (LIFE13 NAT/PT/000458), a contribution of the LIFE financial instrument of the European Union and the "Fundo de Biodiversidade" funded from the Portuguese Government.

8. References

Abrantes, J., Lopes, A.M., Dalton, K.P., Melo, P., Correia, J.J., Ramada, M., Alves, P.C., Parra, F., Esteves, P.J., 2013. New variant of rabbit hemorrhagic disease virus, Portugal, 2012-2013. Emerg. Infect. Dis. 19, 1900–2. doi:10.3201/eid1911.130908

Abrantes, J., van der Loo, W., Le Pendu, J., Esteves, P.J., 2012. Rabbit haemorrhagic disease (RHD) and rabbit haemorrhagic disease virus (RHDV): a review. Vet. Res. 43, 12. doi:10.1186/1297-9716-43-12

Alda, F., Gaitero, T., Suárez, M., Merchán, T., Rocha, G., Doadrio, I., 2010. Evolutionary history and molecular epidemiology of rabbit haemorrhagic disease virus in the Iberian Peninsula and Western Europe. BMC Evol. Biol. 10, 347. doi:10.1186/1471-2148-10-347

Almeida, T., Lopes, A.M., Magalhães, M.J., Neves, F., Pinheiro, A., Gonçalves, D., Leitão, M., Esteves, P.J., Abrantes, J., 2015. Tracking the evolution of the G1/RHDVb recombinant strains introduced

from the Iberian Peninsula to the Azores islands, Portugal. Infect. Genet. Evol. 34, 307–13. doi:10.1016/j.meegid.2015.07.010

Amado, A.; Gafeira, C.; Teixeira, A.; Preto, A. Relatório do Plano de Ordenamento da Reserva Natural das Berlengas. (2007) Instituto de Conservação da Natureza e Florestas , (in Portuguese)

Aragão, H.B., 1927. Myxoma of rabbits. Mem. Inst. Oswaldo Cruz 20, 237-247

Argüello Villares, J., Llanos Pellitero, A., Pérez-Ordoyo Garcia, L., 1988. Enfermedad vírica hemorragica canejo en España. Rev. Med. Vet 5, 645–650.

Asgari, S., Hardy, J.R., Sinclair, R.G., Cooke, B.D., 1998. Field evidence for mechanical transmission of rabbit haemorrhagic disease virus (RHDV) by flies (Diptera:Calliphoridae) among wild rabbits in Australia. Virus Res. 54, 123–32.

Baily, J.L., Dagleish, M.P., Graham, M., Maley, M., Rocchi, M.S., 2014. RHDV variant 2 presence detected in Scotland. Vet. Rec. 174, 411–411. doi:10.1136/vr.g2781

Bertó-Moran, A., Pacios, I., Serrano, E., Moreno, S., Rouco, C., 2013. COCCIDIAN AND NEMATODE INFECTIONS INFLUENCE PREVALENCE OF ANTIBODY TO MYXOMA AND RABBIT HEMORRHAGIC DISEASE VIRUSES IN EUROPEAN RABBITS. J. Wildl. Dis. 49, 10–17. doi:10.7589/2011-12-343

Brouat, C., Tollenaere, C., Estoup, A., Loiseau, A., Sommer, S., Soanandrasana, R., Rahalison, L., Rajerison, M., Piry, S., Goodman, S.M., Duplantier, J.-M., 2014. Invasion genetics of a human commensal rodent: the black rat Rattus rattus in Madagascar. Mol. Ecol. 23, 4153–4167. doi:10.1111/mec.12848

Brugman, V.A., Hernández-Triana, L.M., Prosser, S.W.J., Weland, C., Westcott, D.G., Fooks, A.R., Johnson, N., 2015. Molecular species identification, host preference and detection of myxoma virus in the Anopheles maculipennis complex (Diptera: Culicidae) in southern England, UK. Parasit. Vectors 8, 421. doi:10.1186/s13071-015-1034-8

Cabral, M.J. (coord.); J. Almeida, P.R. Almeida, T. Delliger, N. Ferrand de Almeida, M.E. Oliveira, J.M. Palmeirim, A.I. Queirós, L. Rogado, M. Santos-Reis (eds.) (2005). Livro Vermelho dos Vertebrados de Portugal. Instituto da Conservação da Natureza. Lisboa. 659p. https://dspace.uevora.pt/rdpc/handle/10174/6006?locale=pt

Camarda, A., Pugliese, N., Cavadini, P., Circella, E., Capucci, L., Caroli, A., Legretto, M., Mallia, E., Lavazza, A., 2014. Detection of the new emerging rabbit haemorrhagic disease type 2 virus (RHDV2) in Sicily from rabbit (Oryctolagus cuniculus) and Italian hare (Lepus corsicanus). Res. Vet. Sci. 97, 642–5. doi:10.1016/j.rvsc.2014.10.008

Cancellotti, F.M., Renzi, M., 1991. Epidemiology and current situation of viral haemorrhagic disease of rabbits and the European brown hare syndrome in Italy. Rev. Sci. Tech. 10, 409–22.

Carvalho, C.L., Rodeia J., Branco S., Monteiro M., Duarte E.L., Melo P., Santos P.T., Henriques A.M., Barros S.S., Ramos F., Fagulha T., Fevereiro M., Duarte M.D., 2016. Tracking the Origin of a Rabbit Haemorrhagic Virus 2 Outbreak in a Wild Rabbit Breeding Centre in Portugal; Epidemiological and Genetic Investigation. J Emerg Infect Dis 1: 114. doi:10.4172/24724998.1000114

Catry P, Costa H, Elias G & Matias R (2010). Aves de Portugal, Ornitologia do Território Continental. Assírio e Alvim, Lisboa

Ceia, F., Paiva, V., Fidalgo, V., Morais, L., Baeta, A., Crisóstomo, P., Mourato, E., Garthe, S., Marques, J., Ramos, J., 2014. Annual and seasonal consistency in the feeding ecology of an opportunistic species, the yellow-legged gull Larus michahellis. Mar. Ecol. Prog. Ser. 497, 273–284. doi:10.3354/meps10586

Cooke, B.D., 2002. Rabbit haemorrhagic disease: field epidemiology and the management of wild rabbit populations. Rev. Sci. Tech. 21, 347–58.

Dalton, K.P., Nicieza, I., Balseiro, A., Muguerza, M.A., Rosell, J.M., Casais, R., Álvarez, Á.L., Parra, F., 2012. Variant rabbit hemorrhagic disease virus in young rabbits, Spain. Emerg. Infect. Dis. 18, 2009–12. doi:10.3201/eid1812.120341

Delibes-Mateos, M., Delibes, M., Ferreras, P., Villafuerte, R., 2008. Key role of European rabbits in the conservation of the Western Mediterranean basin hotspot. Conserv. Biol. 22, 1106–17. doi:10.1111/j.1523-1739.2008.00993.x

Duarte, M., Henriques, M., Barros, S.C., Fagulha, T., Ramos, F., Luís, T., Fevereiro, M., Benevides, S., Flor, L., Barros, S.V., Bernardo, S., 2015a. Detection of RHDV variant 2 in the Azores. Vet. Rec. 176, 130. doi:10.1136/vr.h497

Duarte, M.D., Carvalho, C.L., Barros, S.C., Henriques, A.M., Ramos, F., Fagulha, T., Luís, T., Duarte, E.L., Fevereiro, M., 2015b. A real time Taqman RT-PCR for the detection of rabbit hemorrhagic disease virus 2 (RHDV2). J. Virol. Methods 219, 90–5. doi:10.1016/j.jviromet.2015.03.017

Duarte, M., Carvalho, C., Bernardo, S., Barros, S.V., Benevides, S., Flor, L., Monteiro, M., Marques, I., Henriques, M., Barros, S.C., Fagulha, T., Ramos, F., Luís, T., Fevereiro, M., 2015c. Rabbit haemorrhagic disease virus 2 (RHDV2) outbreak in Azores: Disclosure of common genetic markers and phylogenetic segregation within the European strains. Infect. Genet. Evol. 35, 163–71. doi:10.1016/j.meegid.2015.08.005

Duarte, M.D., Henriques, A.M., Barros, S., Luís, T., Fagulha, T., Ramos, F., Fevereiro, M., 2014a. New insight into the epidemiology of rabbit hemorrhagic disease viruses in Portugal: retrospective study reveals the circulation of genogroup 5 (G5) in Azores and discloses the circulation of G1 and G6 strains in mainland until 2008. Infect. Genet. Evol. 27, 149–55. doi:10.1016/j.meegid.2014.07.017

Duarte, M.D., Barros, S.C., Henriques, A.M., Fagulha, M.T., Ramos, F., Luís, T., Fevereiro, M., 2014b. Development and validation of a real time PCR for the detection of myxoma virus based on the diploid gene M000.5L/R. J. Virol. Methods 196, 219–24. doi:10.1016/j.jviromet.2013.11.014

Dunnet, G., Dunnet, G., 1956. Growth rate of young rabbits, Oryctolagus cuniculus (L.). CSIRO Wildl. Res. 1, 66. doi:10.1071/CWR9560066

Fenner, F., Ratcliffe, F.N., 1965. Myxomatosis. Cambridge University Press, London

Gomes, C.T, Draper, D., Marques, I., Rosello-Graell, A., 2004. Componente Vegetal do Plano de Ordenamento da Reserva Natural das Berlengas: Flora e vegetação do arquipélago das Berlengas. Unpublished report

Gonçalves, H., Alves, P.C., Rocha, A., Gonçalves, H., Alves, P.C., Rocha, A., 2002. Seasonal variation in the reproductive activity of the wild rabbit (Oryctolagus cuniculus algirus) in a Mediterranean ecosystem. Wildl. Res. 29, 165. doi:10.1071/WR00048

Hall, R.N., Mahar, J.E., Haboury, S., Stevens, V., Holmes, E.C., Strive, T., 2015. Emerging Rabbit Hemorrhagic Disease Virus 2 (RHDVb), Australia. Emerg. Infect. Dis. 21, 2276–8. doi:10.3201/eid2112.151210

Henning, J., Meers, J., Davies, P.R., Morris, R.S., 2005. Survival of rabbit haemorrhagic disease virus (RHDV) in the environment. Epidemiol. Infect. 133, 719–30.

Huelsenbeck, J.P., Ronquist, F., Nielsen, R., Bollback, J.P., 2001. Bayesian inference of phylogeny and its impact on evolutionary biology. Science 294, 2310–4. doi:10.1126/science.1065889

Kerr, P.J., 2012. Myxomatosis in Australia and Europe: A model for emerging infectious diseases. Antiviral Res. 93, 387–415. doi:10.1016/j.antiviral.2012.01.009

Kerr, P.J., Liu, J., Cattadori, I., Ghedin, E., Read, A.F., Holmes, E.C., 2015. Myxoma virus and the Leporipoxviruses: an evolutionary paradigm. Viruses 7, 1020–61. doi:10.3390/v7031020

Le Gall, G., Arnauld, C., Boilletot, E., Morisse, J.P., Rasschaert, D., 1998. Molecular epidemiology of rabbit haemorrhagic disease virus outbreaks in France during 1988 to 1995. J. Gen. Virol. 79 (Pt 1), 11–6. doi:10.1099/0022-1317-79-1-11

Le Gall-Reculé, G., Lavazza, A., Marchandeau, S., Bertagnoli, S., Zwingelstein, F., Cavadini, P., Martinelli, N., Lombardi, G., Guérin, J.-L., Lemaitre, E., Decors, A., Boucher, S., Le Normand, B., Capucci, L., 2013. Emergence of a new lagovirus related to Rabbit Haemorrhagic Disease Virus. Vet. Res. 44, 81. doi:10.1186/1297-9716-44-81

Le Gall-Reculé, G., Zwingelstein, F., Boucher, S., Le Normand, B., Plassiart, G., Portejoie, Y., Decors, A., Bertagnoli, S., Guérin, J.-L., Marchandeau, S., 2011. Detection of a new variant of rabbit haemorrhagic disease virus in France. Vet. Rec. 168, 137–8. doi:10.1136/vr.d697

Licois, D., 2004. Domestic rabbit enteropathies, in: Proc. 8th World Rabbit Congress, Puebla, Mexico. pp. 385–403

Liu S.J., Xue H.P., Pu B.Q., Qian N.H., 1984. A new viral disease of rabbits. Animal Husbandry Vet Med.16, 253-255

Lopes, A.M., Correia, J., Abrantes, J., Melo, P., Ramada, M., Magalhães, M.J., Alves, P.C., Esteves, P.J., 2015. Is the new variant RHDV replacing genogroup 1 in Portuguese wild rabbit populations? Viruses 7, 27–36. doi:10.3390/v7010027

Maddison WP, Maddison D.R. (2009) Mesquite: a modular system for evolutionary analysis. Version 2.72 http://mesquiteproject

Marcato, P.S., Benazzi, C., Vecchi, G., Galeotti, M., Della Salda, L., Sarli, G., Lucidi, P., 1991. Clinical and pathological features of viral haemorrhagic disease of rabbits and the European brown hare syndrome. Rev. Sci. Tech. 10, 371–92

Martin-Alonso, A., Martin-Carrillo, N., Garcia-Livia, K., Valladares, B., Foronda, P., 2016. Emerging rabbit haemorrhagic disease virus 2 (RHDV2) at the gates of the African continent. Infect. Genet. Evol. 44, 46–50. doi:10.1016/j.meegid.2016.06.034

McColl, K.A., Merchant, J.C., Hardy, J., Cooke, B.D., Robinson, A., Westbury, H.A., 2002. Evidence for insect transmission of rabbit haemorrhagic disease virus. Epidemiol. Infect. 129, 655–63.

McIntosh, M.T., Behan, S.C., Mohamed, F.M., Lu, Z., Moran, K.E., Burrage, T.G., Neilan, J.G., Ward, G.B., Botti, G., Capucci, L., Metwally, S.A., 2007. A pandemic strain of calicivirus threatens rabbit industries in the Americas. Virol. J. 4, 96. doi:10.1186/1743-422X-4-96

Meirinho A, Barros N , Oliveira N , Catry P, Lecoq M, Paiva V, Geraldes P, Granadeiro JP, Ramírez I & Andrade J (2014). Atlas das Aves Marinhas de Portugal. Sociedade Portuguesa para o Estudo das Aves

Merchán, T., Rocha, G., Alda, F., Silva, E., Thompson, G., de Trucios, S.H., Pagés, A., 2011. Detection of rabbit haemorrhagic disease virus (RHDV) in nonspecific vertebrate hosts sympatric to the

European wild rabbit (Oryctolagus cuniculus). Infect. Genet. Evol. 11, 1469–74. doi:10.1016/j.meegid.2011.05.001

Muller, A., Freitas, J., Silva, E., Le Gall-Reculé, G., Zwingelstein, F., Abrantes, J., Esteves, P.J., Alves, P.C., van der Loo, W., Kolodziejek, J., Nowotny, N., Thompson, G., 2009. Evolution of rabbit haemorrhagic disease virus (RHDV) in the European rabbit (Oryctolagus cuniculus) from the Iberian Peninsula. Vet. Microbiol. 135, 368–73. doi:10.1016/j.vetmic.2008.09.057

Musser GG, Carlton MD. Superfamily Muroidea. In: Wilson DE, Reeder DM, eds. Mammal Species of the World, 3rd ed. Baltimore, MD: The Johns Hopkins University Press, 2005:894–1531

Nowotny, N., Bascuñana, C.R., Ballagi-Pordány, A., Gavier-Widén, D., Uhlén, M., Belák, S., 1997. Phylogenetic analysis of rabbit haemorrhagic disease and European brown hare syndrome viruses by comparison of sequences from the capsid protein gene. Arch. Virol. 142, 657–73

OIE. 2016. Terrestrial manual. Chapter 2.6.2. Rabbit haemorrhagic disease. < http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.06.02_RHD.pdf

PFMA (Pet Food Manufacturers Association. (2012). Rabbit Size-O-Meter. https://research.unc.edu/files/2012/11/Body-Condition-Scoring-Rabbit.pdf

Pinto, Gabriela A., 1995- Contribuição para o Estudo Bioecológico dos Ectoparasitas do Coelho (Oryctolagus cuniculus L. 1758) e do Rato-preto (Rattus rattus L. 1758) na Reserva Natural da Berlenga. Relatório entregue ao Instituto da Conservação da Natureza, 15pp

Puggioni, G., Cavadini, P., Maestrale, C., Scivoli, R., Botti, G., Ligios, C., Le Gall-Reculé, G., Lavazza, A., Capucci, L., 2013. The new French 2010 Rabbit Hemorrhagic Disease Virus causes an RHD-like disease in the Sardinian Cape hare (Lepus capensis mediterraneus). Vet. Res. 44, 96. doi:10.1186/1297-9716-44-96

Ronquist, F., Huelsenbeck, J.P., 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19, 1572–4

Sanarelli, G., 1898. Das myxomatogene Virus. Beitrag zum Stadium der Krankheit-serreger ausserhalbdes Sichtbaren. Zbl. Bakt 23, 865-873

Sievers, F., Wilm, A., Dineen, D., Gibson, T.J., Karplus, K., Li, W., Lopez, R., McWilliam, H., Remmert, M., Söding, J., Thompson, J.D., Higgins, D.G., 2011. Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. Mol. Syst. Biol. 7, 539. doi:10.1038/msb.2011.75

Silva, S.M., Ferreira, C., Paupério, J., Silva, R.M., Alves, P.C., Lemos, A., 2015. Coccidiosis in European rabbit (Oryctolagus cuniculus algirus) populations in the Iberian Peninsula. Acta Parasitol. 60, 350–355. doi:10.1515/ap-2015-0049

Southern, H.N., 1940. THE ECOLOGY AND POPULATION DYNAMICS OF THE WILD RABBIT (ORYCTOLAGUS CUNICULUS). Ann. Appl. Biol. 27, 509–526. doi:10.1111/j.1744-7348.1940.tb07522.x

Tavaré, S., 1986. Some probabilistic and statistical problems in the anal- ysis of DNA sequences. In: Miura R. M., (Ed). Lectures on math- ematics in the life sciences. Volume 17. Providence (RI):American Mathematical Society. pp. 57–86

Tham, K.M., Barnes, S.M., Hunter, S.N., 1999. Polymerase chain reaction amplification and gene sequence analysis of a calicivirus from a feral rabbit. Virus Genes 18, 235–42

Vicente, L. A., 1989- Sobre a História Natural dos Répteis da Ilha Berlenga – A síndrome de Insularidade. Dissertação de Doutoramento. Faculdade de Ciências , Universidade de Lisboa, 487pp

Westcott, D.G., Frossard, J.-P., Everest, D., Dastjerdi, A., Duff, J.P., Choudhury, B., 2014. Incursion of RHDV2-like variant in Great Britain. Vet. Rec. 174, 333–333. doi:10.1136/vr.g2345

Chapter V

RHDV and RHDV2 genetic relations

1. Summary on the phylogenetic and evolutionary methods used

To better conduct the reader in the comprehension of this chapter and of the studies included within it, a summary on the methods used for phylogenetic analysis will be present next.

1.1 Phylogenetic analysis

The widespread use of phylogenies is a consequence of their importance to answer questions in biology, of the immense quantity of sequence data that have been produced and of the fact that sophisticated analysis can be currently performed by fast computers (Huelsenbeck et al., 2001). The reconstruction of phylogenetic trees is an utmost tool to study evolution and the most commonly used methodology to depict the historical associations of life (Saitou and Imanishi, 1989). A phylogenetic tree is a branch-like structure that illustrates ancestor–descendent relationships among organisms (Kong, 2015).

A wide variety of inference methods that are actively propounded by different experts can be used for constructing phylogenetic trees from molecular data (Saitou and Imanishi, 1989)(Huelsenbeck et al., 2001).

Estimating phylogenetic trees by, at least, two methods, adds confidence to the resulting analysis if the same results are obtained (Mount, 2008). The performance of phylogenetic methods is usually evaluated regarding their consistency (the ability to estimate the correct phylogeny with sufficient data), efficiency (the ability to quickly converge on the correct phylogeny), and robustness (the ability to estimate the correct phylogeny even when the assumptions of the phylogenetic method are violated). The latter, robustness, may be the most important criteria because the assumptions underlying phylogenetic methods are most likely violated with real data, and the ability to estimate a phylogeny correctly regardless of model violation is very important (Huelsenbeck, 1995).

Molecular phylogenetics infers phylogenetic relationships from molecular sequence data to deduce the history that is more consistent with a set of observed data (Swofford et al., 1996). Sequences for phylogenetic analysis can be nucleotide or protein sequences which should align with each other along their entire lengths or, at least, have a common set of domains that indicate evolutionary relatedness (Mount, 2008). A phylogenetic analysis should be performed when the sequences produce a multiple sequence alignment (msa) in which their similarity is apparent by the presence of conserved positions (Mount, 2008).

Various evolutionary forces act on DNA sequences resulting in sequences change over time (Strimmer and von Haeseler, 2009). Any two sequences derived from a common ancestor evolving independently of each other eventually diverge and a measure of this divergence is called genetic distance, which plays an important role in many aspects of sequence analysis (Strimmer and von Haeseler, 2009). The computation of a matrix of genetic or evolutionary distances is one of the first steps of sequences analysis and calculates for every pair of sequences an estimate of the branch length separating them, where branch length is the product of time and rate of evolution (Strimmer and von Haeseler, 2009).

The results of any phylogenetic analysis are conditional on the assumptions made in the analysis and the specification of the most appropriate model that fits the data, providing a statistical description of the DNA substitution rates (Huelsenbeck et al., 2001). Modelling assumptions that poorly fit the observations can lead to erroneous inferences (Huelsenbeck et al., 2001). If an evolutionary model accurately explains the observed DNA sequences, then data simulated under that model should be similar to the observations (Huelsenbeck et al., 2001).

1.1.1. Models of sequence evolution (DNA substitution)

Models of nucleotide or amino acid evolution are essential in the analysis of molecular sequence data (Swofford et al., 1996). They are tools that reduce the enormous complexity of the biological mutation process to a comparatively simple pattern that can be described by a small number of parameters (Strimmer, 1997). They specify a modus of substitution for nucleotides or amino acids at a given site and determine how the rate of substitutions is distributed over different positions in a sequence (model of rate heterogeneity) (Strimmer, 1997).

The mathematical expression of a substitution model is a table of rates (substitution per site per unit evolutionary distance) in which each nucleotide is replaced by each alternative nucleotide (Swofford et al., 1996). A DNA sequence is composed by four nucleotides adenine (A), cytosine (C), guanine (G), and thymine (T), existing $n = 4$ different states for a sequence position (Swofford et al., 1996). For DNA sequences, the nucleotide substitution process can be expressed or generalized by the Markov process that uses a 4 x 4 instantaneous rate matrix, the instantaneous rate matrix Q, in which each element represents the rate of change from nucleotide i to j during a time period (Strimmer and Haesler, 2009). The models summarized by the Q matrix fit in the class of models known as time-homogeneous time continuous stationary Markov models, all sharing three assumptions. The first is the Markov property meaning that, at any given site in a sequence, the

rate of change from base *i* to base *j* is independent from the base that occupied that site prior *i*. The second is homogeneity, meaning that substitution rates do not change over time and the third is stationarity, the relative frequencies of A, C, G and T are at equilibrium (Strimmer and Haesler, 2009).

Almost all of the DNA substitution models proposed are special cases of the Q matrix (Swofford et al., 1996). Within this general framework, several sub-models can be developed as the so-called time-reversible models, such as the most general time-reversible model (GTR) (Tavaré et al., 1986), which assume that for any two nucleotides the rate of change from *i* to *j* is always the same than from *j* to *i* (a = g, b = h, c = i, d = j, e = k, f = g in the Q matrix) (Swofford et al., 1996)(Strimmer and von Haesler, 2009). Once the Q matrix and the evolutionary model are specified, it is possible to calculate the probabilities of change from any base to any other during the evolutionary time by computing the matrix exponential (Strimmer and von Haesler, 2009). As reviewed by Swofford et al. (1996), most of the remaining models commonly used for maximum likelihood (ML) tree inference or estimation of pairwise evolutionary distances, can be obtained by restricting the parameters of the GTR matrix. The model of Tamura and Nei 1993 (TN93) is obtained by requiring that a = c = d = f, if the substitution types are divided into transversions, transitions between purines, and transitions between pyrimidines. Further restrictions on the GTR matrix parameters lead to other familiar models. Assuming that the equilibrium frequencies of all bases are the same and that all substitutions occur at the same rate, the model reduces to that of Jukes and Cantor 1969 (JC). The Kimura two-parameter model (K2P, 1980) considers that transitions and transversions occur at different rates, but still assumes equal base frequencies. This model (K2P) can be easily generalized to allow unequal equilibrium base frequencies giving rise to the Hasegawa (1985) model (HKY85), corresponding to the GTR model with the certain mathematical constraints. Likewise, the JC model can be generalised to allow for unequal base frequencies giving rise to the Felsenstein (1981) model (F81), also described as the "equal output" model by Tajima and Nei (1982). Felsenstein (1984) used a different model to accommodate unequal base frequencies in a two parameter model (the F84 model, formally described in Kishino and Hasegawa, 1989). The F84 model divides the substitution process into two components, a general substitution rate capable of producing all types of substitutions, and a within-group substitution rate that produces only transitions.

1.1.2. Methods for investigating the temporal signal and molecular clock of phylogenies

Genetic sequences are designated 'heterochronous', or measurably evolving, if obtained at evolutionarily distinct points in time (Rambaut et al., 2016). Two sampling times are considered 'evolutionarily distinct' if the corresponding genetic sequences differ by a measurable amount of nucleotide or amino acid substitution (Drummond et al., 2003).

The sampling dates of heterochronous sequences contain information on the rate of sequence evolution and can be used to directly infer molecular phylogenies whose branch lengths represent time, the 'time trees' or 'clock trees' (Rambaut et al., 2016). Time-scaled trees can be estimated using several statistical approaches, such as Bayesian inference (Drummond et al., 2012), maximum likelihood (Rambaut, 2000)(Sanderson, 2003)(Yang, 2007), or heuristic methods (Drummond and Rodrigo, 2000). However, a molecular clock model, consisting of a statistical description of the relationship between observed genetic distances and time is required (Rambaut et al., 2016). Such models that allow the rate of evolution to vary among the branches of a phylogenetic tree have been developed and are generally referred to as relaxed or local molecular clocks (Huelsenbeck et al., 2000)(Kishino et al., 2001)(Drummond et al., 2006)(Drummond and Suchard, 2010). According to Rambaut et al. (2016), for the reliable estimation of a time-scaled tree it is important to confirm that the sequences contain a satisfactory 'temporal signal' meaning that there must be sufficient genetic change between sampling times to reconstruct a statistical relationship between genetic divergence and time. Exploring the degree of temporal signal in heterochronous sequences can be achieved using a simple regression-based approach. Regression of root-to-tip genetic distance against sampling time can be used as a simple diagnostic tool for molecular clock models. A linear trend with small residuals indicates that evolution will be adequately represented by a strict molecular clock. The same trend with greater scatter from the regression line suggests that a relaxed molecular clock model may be more appropriate. A strong non-linear trend suggests that evolutionary rate has systematically changed through time, and no trend indicates that the data contain little temporal signal and are unsuitable for inference using phylogenetic molecular clock models. The regression should be used as a data exploration tool rather than for formal hypothesis testing.

1.1.2.1. The TempEst software

The program TempEst (Temporal Exploration of Sequences and Trees) is a tool for investigating the temporal signal and 'clocklikeness' of molecular phylogenies proposed by Rambaut et al. (2016). The software is a cross-platform, open source, graphical program for exploring heterochronous data, freely available from http://tree. bio.ed.ac.uk/. During its development, it was formerly known as “Path-O-Gen”. As input, TempEst takes a ‘non-clock’ phylogenetic tree (whose branch lengths are genetic distances), estimated using neighbor-joining (NJ), maximum likelihood (ML), or Bayesian approaches. Once loaded, the user provides sampling dates or ages for each sequence.

In brief, the TempEst software can read and analyse contemporaneous trees for which all sequences were collected at the same time, and dated-tip trees for which sequences were collected at different dates, and that have not been inferred under a molecular-clock assumption to see how valid this assumption may be. It can also root the tree at the position that is likely to be the most compatible with the assumption of the molecular clock.

1.1.3. Methods estimating phylogenetic trees

Statistical methods to estimate evolutionary trees and test hypotheses on the evolutionary process are fundamental (Felsenstein, 1981) and there is a wide variety of inference methods proposed by different experts (Huelsenbeck et al., 2001).

Maximum parsimony (MP) is a popular technique for phylogeny reconstruction (Steel and Penny, 2000) and assumes that change is improbable *a priori* (Felsenstein, 1973). If, over the evolutionary time considered, the amount of change is small, parsimony methods are adequate statistical methods (Felsenstein, 1981). However, most data involve moderate to large amounts of change, and parsimony methods can be a statistically unsound producing an inconsistent estimate of the evolutionary tree (Felsenstein, 1981).

Reconstruction methods based on more realistic models of molecular evolution became available (Douady et al., 2003) and model-based approaches have come to rival MP, and even dominate phylogenetic methodology (Steel and Penny, 2000). Even when parsimony is consistent, other methods incorporating models of evolutionary change make more effective use of the data (Swofford et al., 1996). Maximum likelihood (ML) is one of the most used alternatives (Steel and Penny, 2000). While MP seeks solutions that minimize the amount of evolutionary change required to explain the data, ML attempt to estimate the actual amount of change according to an evolutionary model (Swofford et al., 1996).

Other approaches may include distance-based methods that use transformed or inferred distances (Steel and Penny, 2000), reflecting the mean number of changes per site that have occurred between a pair of sequences since their divergence from a common ancestor (Swofford et al., 1996). There are several distance matrix methods one of the most common is neighbor-joining (Saitou and Nei, 1987). Overall, under the same conditions, likelihood methods outperform distance methods in choosing the correct tree (Hueselnbeck, 1995).

More recently, phylogeny Bayesian inference brought a new perspective to a number of issues in evolutionary biology, including the analysis of large phylogenetic trees, complex evolutionary models and the detection of the effects of natural selection in DNA sequences (Hueselnbeck, 2001). According to the strategy used for finding the best tree, methods for estimating phylogenetic trees can be classified into two categories. The "exhaustive-search" method, that consists in examining all or a large number of possible trees, often using the principle of minimum evolution or maximum parsimony (Saitou and Nei, 1987)(Saitou and Imanishi, 1989). The maximum-parsimony (MP) method and the maximum-likelihood (ML) method fit in this category. The "stepwise clustering" method consists in examining local topological relationships of a tree and construct the best tree step-by-step. The neighbor-joining (NJ) method and many other distance methods belong to this category) (Saitou and Imanishi, 1989).

1.1.3.1. The neighbor-joining (NJ) method

The neighbor-joining (NJ) method was proposed by Saitou and Nei (1987) and estimates phylogenetic trees from evolutionary distance data (Saitou and Nei, 1987). The principle of the NJ method is to find pairs of operational taxonomic units (OTUs or neighbours) that minimize the total branch length at each stage of OTUs clustering, starting with a starlike tree (Saitou and Nei, 1987). A pair of neighbours is a pair of OTUs connected through a single interior node in an unrooted, bifurcating tree (Saitou and Nei, 1987).

According to Saitou and Nei (1987), the NJ method produces a unique final tree under the principle of minimum evolution and is applicable to any type of evolutionary distance data. The algorithm of the NJ method provides the topology and the branch lengths of the final tree. Unlike the standard algorithm for minimum-evolution trees, the NJ method minimizes the sum of branch lengths at each stage of OTUs clustering. Hence, the final tree produced may not be the minimum-evolution tree among all possible trees. The method is particularly useful when the number of sequences under analysis is in the order of hundreds or thousands. The NJ method constructs trees by

clustering neighbouring sequences in a stepwise manner. In each step of sequence clustering, it minimizes the sum of branch lengths and thus examines multiple topologies. However, for large data sets, NJ examines only a small fraction of the total number of possible topologies. Despite the NJ method is considered statistically consistent, if correct pairwise distances with no statistical errors are used, reconstructing the true tree, in practice, estimates of all distances are subject to statistical errors, producing eventually erroneous trees. Pairwise distances used for constructing NJ trees are estimated by the IE (independent estimation) method for a variety of mathematical models to incorporate varying degrees of complexity of nucleotide or amino acid substitution. The estimates obtained by the IE method are expected to have larger standard errors than those obtained by the SE (simultaneous estimation) method (Tamura et al., 2004)(Saitou and Nei, 1987). The evolutionary distance based on the TN93 model (Tamura and Nei, 1993) is one of the most sophisticated models of nucleotide substitution.

1.1.3.2. Maximum Likelihood

Felsenstein (1981) proposed a maximum likelihood (ML) method for inferring evolutionary trees using discrete characters such as nucleotide sequences (Felsenstein, 1981).

As reviewed by Swofford et al. (1996), ML methods are conceptually simple and evaluate a hypothesis in terms of the probability that a proposed model of the evolutionary process and the hypothesized history would give rise to the observed data.

To apply a ML approach, a concrete model of the evolutionary process responsible for the conversion of one sequence into another must be specified. Phylogenies are then inferred by finding the trees that yield the highest likelihoods (Sowfford et al., 1996). A Markov model is used to describe the evolutionary changes between character states (Rannala and Yang, 1996). Assuming that nucleotide sites evolve independently, the likelihood for each site can be calculated separately and the likelihood combined into a total value at the end. Having calculated likelihoods at each site, the joint probability that the tree and model confer upon all sites is computed as the product of the individual sites likelihoods (Swofford et al., 1996). The tree topology and branch lengths are treated as parameters (Rannala and Yang, 1996). Branch lengths and parameters in the substitution model are estimated by ML for each tree topology, generating the maximum likelihood value for that topology. The tree with the highest (maximum) likelihood is chosen as the best estimate of phylogeny (Rannala and Yang, 1996). Because the probability of any single observation is an extremely small number, the log of the likelihood is evaluated instead, so the probabilities are the

sum of the logs of the single-site likelihoods (Sowfford et al., 1996). A nonparametric bootstrapping method is used for evaluating the estimated tree significance (Felsenstein, 1985).

In brief, a maximum-likelihood analysis consists of three parts. First, a model of evolutionary change for nucleotides or amino acids is specified. Then, based on this model, different hypotheses about the evolutionary history are evaluated in terms of the probability that the hypothesized history would give rise to the observed data. Finally, the hypothesis is selected that shows the highest probability (Swofford et al., 1996).

When implemented with the same models of DNA substitution, ML outperformed NJ methods, confirming its general superiority under comparable conditions (Huelsenbeck, 1995).

Due to its consistency, the ML method yields estimates that frequently have lower variance than other methods, and is often the estimation method least affected by sampling error (Petersen and Deddens, 2008). It also tends to be more robust in face of the models assumptions violations (Huelsenbeck, 1995). In fact, part of the ML method strength is that many models of sequence evolution, assuming identical distributions across sites, can safely assume that the substitution process at different sites have much in common, even if they are not exactly identical. Therefore, the major components influencing the evolution of sequences can be determined by just a few parameters (Swofford et al., 1996).

1.1.3.3. Bayesian inference of phylogeny

A Bayesian method for estimating phylogenetic trees was proposed by Rannala and Yang (1996) based on a quantity called the posterior probability of a tree, which is the probability that the tree is correct (Huelsenbeck et al., 2001). The Bayes's theorem is used to combine the prior probability of a phylogeny with the likelihood to produce a posterior probability distribution on trees (Huelsenbeck et al, 2001).

Rannala and Yang (1996) used a birth-death process (a continuous-time process that calculates the probability of a speciation event) to specify the prior distribution of tree topologies and divergence times (branch lengths) of the species under analysis and a continuous-time Markov process to model nucleotide substitution. For both processes, the parameters were estimated by maximum likelihood, *i.e.* the probability of observing the data. The posterior probability of each tree topology, conditional on the nucleotide sequence data and the estimated parameters, is then calculated. The best estimate of the phylogeny can be selected as the tree with the highest

posterior probability, the maximum posterior probability (MAP) tree, representing the best estimate of the evolutionary relationships among species (Rannala and Yang, 1996).
In the Bayesian inference proposed by Rannala and Yang (1996) topologies and branch lengths are treated as random variables rather than parameters.
The posterior probabilities are a measure of the reliability of the estimated phylogeny but involve a summation over all trees and, for each tree, integration over all possible combinations of branch length and substitution model parameter values and cannot be obtained analytically (Huelsenbeck et al, 2001). Hence, they must be approximated by numerical methods, of which the Markov chain Monte Carlo (MCMC) was proven the most useful (Yang and Rannala, 1997)(Huelsenbeck et al, 2001). The MCMC method has revolutionized Bayesian inference (Huelsenbeck et al., 2001) and refers to the construction of a Markov chain designed to explore the posterior probability surface by integration over the space of model parameters. Trees are sampled at fixed intervals and the posterior probability of a given tree is approximated by the proportion of time that the chain visited it (Yang and Rannala, 1997)(Huelsenbeck et al., 2001). This means that the current tree is stochastically perturbed and a new tree is proposed, which is then accepted or rejected with a probability described by Metropolis et al. (1953) and Hastings (1970). If the new tree is accepted, then it is subject to further perturbation (Huelsenbeck et al., 2001). By evaluating the posterior probabilities of trees, a MCMC method visits only a small portion of all possible trees, avoiding the need to sum over all topologies (Yang and Rannala, 1997). A consensus tree is obtained from these sampled trees, and Bayesian posterior probabilities (PP) of individual clades, as expressed by the consensus indices, represent the clade credibility values (Douady et al., 2003).
Bayesian analysis of the initial matrix of taxa and characters generates a MAP tree and estimates of uncertainty of its nodes, directly assessing the substitution model, branch length, and topological variables, as well as clade reliability values, all in a reasonable computation time (Douady et al., 2003).

1.1.3.3.1. Posterior probabilities (PP) and nonparametric bootstrapping (BS)

Both posterior probabilities (PP) and bootstrap (BS) supports are of great interest to phylogeny as potential upper and lower bound of node support, but they are not interchangeable and cannot be directly compared (Douady et al., 2003).
With Bayesian methods, reliability of the MAP tree nodes derives directly from corresponding PP (Yang and Rannala, 1997). Statistically, posterior probabilities have the advantage to be of

straightforward interpretation as they represent the probability that the corresponding clade is true, given the model, the priors, and the data (Huelsenbeck et al., 2002). They are not tightly correlated with ML bootstrap percentages as verified by Douady et al. (2003) and seem to be less conservative than BS, in turn an overconservative estimator of node reliability (Douady et al., 2003). Being more conservative, the bootstrap approach might be less prone to strongly support a false phylogenetic hypothesis and apparent conflicts in topology recovered by the Bayesian approach are reduced after bootstrapping (Douady et al., 2003).

1.1.4. Other methods used in phylogenetic analysis

1.1.4.1. Networks

Huson and Bryant (2006) proposed defining a phylogenetic network as any network in which taxa are represented by nodes and their evolutionary relationships by edges, including phylogenetic trees. Modern systematics adopted phylogenetic trees as the integral component of the evolutionary model (Huson and Bryant, 2006). However, phylogenetic trees are branch-like patterns and oversimplification of more complex evolutionary processes have been considered as limitations of the approach (Huson and Bryant, 2006)(Huson et al., 2010)(Huson and Scornavacca, 2010)(Moret et al., 2004)(Posada and Crandall, 2001). Some researchers argue that the multitude of plausible trees is best expressed by a network displaying alternative potential evolutionary paths in the form of cycles (Bandelt et al., 1999) and there has been an increase in the development and use of other phylogenetic networks to represent and analyse evolutionary relationships among organisms (Huson et al., 2010)(Kong, 2015). These phylogenetic networks are a generalization of evolutionary trees (Huber et al., 2015) and consist of connected graphs with cycles representing potentially complex patterns of evolutionary relationship (Huson et al., 2010)(Huson and Scornavacca, 2010)

Several network construction techniques have been proposed, including rooted and un-rooted networks. Although the term ‘phylogenetic network’ is widely used referring to both rooted and un-rooted networks, the adjective ‘phylogenetic’ is unsuitable for un-rooted networks since they do not show direction, *i.e.* ancestor–descendant relationships. On the contrary, rooted networks depict hypotheses of evolutionary change in time, making the term ‘phylogenetic network’ appropriate (Kong, 2015). Furthermore, networks can represent explicit or abstract relationships between taxa (Huson and Scornavacca, 2010)(Huson et al., 2010). Explicit networks describe evolutionary

scenarios once the edges represent the evolutionary history of the nodes. In opposition, abstract networks, in which edges act as linkages, are evolutionarily and biologically uninformative and used as a tool for visualizing incompatible datasets (Kong, 2015). Rooted networks can be abstract or explicit, depending on the algorithm and interpretation, but most, if not all, un-rooted networks are merely abstract networks, that picture conflicts between trees and cannot narrate evolutionary history (Kong, 2015), since evolution is inherently rooted (Huson and Scornavacca, 2010)(Huson et al., 2010).

There are two major types of un-rooted networks, the split networks and the quasi-median networks. The split network attempts to represent only bipartitions or splits in data, emphasizing their distinctive features (such as nucleotide differences), and the evidence that these splits provide for contradictory relationships (Dress and Huson, 2004)(Morrison, 2010). In these networks, internal nodes usually have no explicit meaning (Huber et al., 2015), only providing an implicit picture of evolutionary relationships (Huson and Bryant, 2005). The internal nodes in a split network do not necessarily correspond to hypothetical ancestors (Huson and Bryant, 2006). Quasi-median networks were developed to represent multi-state characters, often creating a network too large and complicated to be practical. To overcome this constraint, Bandelt et al. (1999) developed the median-joining algorithm to construct an informative sub-network of the full quasi-median network, guided by the minimum-spanning network (Kong, 2015). This network is addressed next in point "1.1.4.1.1. Median-joining networks".

Rooted phylogenetic networks include reticulate networks, constructed to model evolutionary history, wherein the evolution is suspected of being reticulate in nature. They provide an explicit picture of evolution once the edges have a direction with an evolutionary meaning (Huson and Bryant, 2006). In fact, the edges represent lineages of descent or reticulate events such as hybridization, horizontal gene transfer, or recombination, and all nodes correspond to hypothetical ancestors (Huber et al., 2015).

1.1.4.1.1. Median-Joining networks

One of the most popular un-rooted networks is the median joining network (MJN) proposed by Bandelt et al. (1999) for phylogeographic inference, increasing in popularity (Kong, 2015).

The MJN algorithm combines the minimum spanning network (MSN) and quasi-median network, creating an intermediate-sized network in the process of overcoming the limitations of both methods (Barthelemy, 1989)(Bandelt, 1992)(Bandelt, 1995)(Kong, 2015). The method produces a

network containing multiple plausible trees in a single reticulating figure giving a more concise picture of the data (Bandelt, 1995) and requires msa with infrequent ambiguous states and the absence of recombination that would produce high-dimensional networks impossible to interpret (Kong, 2015). The MJ method begins with the minimum spanning trees, all combined within a single (reticulate) network (Bandelt, 1995).

The shortcomings of the method are that it relies on distance-based phenetics (overall similarity instead of character transformations) and the lack of rooting (no direction or history). The approach cannot offer defensible evolutionary interpretations considering that evolution involves both change and time, and the lack of rooting removes time from the equation, meaning ancestor–descendant relationships (Kong, 2015).

1.1.4.1.1.1. The Network software

MJN has several applications in evolutionary biology mostly because of the freely available software NETWORK (Bandelt et al., 1999)(Fluxus Technology, 1999; www.fluxus engineering.com). In NETWORK, MJN can be computed with multi-state data (including infrequent ambiguities such as N) in DNA, RNA, amino acid nucleotide sequences, microsatellites (STRs) and endonuclease data (Kong, 2015). Sequences must be aligned correctly and it is stipulated that ambiguous states are infrequent and recombination is absent (Bandelt, 1999). In the displayed graph, each circle represents a unique haplotype whose diameter is proportional to the number of sequences represented. Small solid circles indicate median vectors representing existing un-sampled genotypes or extinct ancestral sequences (Kong, 2015).

The network can predict haplotypes, indicate where homoplasy is located, which sites mutated frequently, where a consensus sequence is, whether recombination is likely to have occurred, where to look for sequence errors, which haplogroups may be discerned, and so on.

1.1.5. The R project for statistical computing

The R software was developed by John Chambers and colleagues at Bell Laboratories (Lucent Technologies). It is a free software environment for wide variety of statistical (e.g. linear and nonlinear modelling, classical statistical tests, time-series analysis, classification, clustering) and graphical techniques, and is highly extensible (r-project.org/about.html; accessed in 17-06-2017). As the software was designed around a true computer language, it allows users to add additional functionality by defining new functions and it can be extended easily via packages, most are

available through the CRAN family of internet sites covering a very wide range of modern statistics. (r-project.org/about.html; accessed in 17-06-2017).

With more than 20 packages devoted to phylogenetics, the R software (R Development Core Team, 2009) has become a standard in phylogenetic analysis (see http://cran.r-project.org/web/views/ Phylogenetics.html for an overview)(Schliep et al., 2011).

2. RHDV and RHDV2 genetic relations

2.1 Pathogenic lagoviruses

Pathogenic strains of RHDV can be divided into three groups, i) the classical RHDV genogroups 1 to 5 (G1–G5) (Nowotny et al., 1997)(Le Gall-Reculé et al., 2003), ii) genogroup G6 (also designated RHDVa due to its distinct antigenic profile (Capucci et al., 1998), and iii) the recently emerged RHDV2, also referred as RHDVb (Le Gall-Reculé et al., 2011a). RHDV and RHDVa are phylogenetically related sharing more than 85% nucleotide similarity with RHDV2 (Le Gall-Reculé et al., 2011a)(Abrantes et al., 2013)(Dalton et al., 2012)(Le Gall-Reculé et al., 2013)(Esteves et al., 2015). RHDV emerged more than three decades ago (Liu et al., 1984), while RHDV2 was reported for the first time in 2010 (Le Gall-Réculé et al., 2011a).

2.1.1. RHDV

2.1.1.1 RHDV origin and evolution

For RHDV, a wide number of phylogenetic studies have been published showing significant discrepancies among substitution rates and a substantial variation of the time span to the most recent common ancestor (TMRCA), placing the RHDV emergence in the past and making the evolutionary history of this virus as well as its geographic origins controversial among researchers (Hicks and Duffy, 2012).

Two major hypotheses have been proposed to explain the origin of RHDV and the emergence of RHDV2: the evolution from pre-existing non-pathogenic virus (NP-LV) circulating in European leporids, and a virus jump to a new host species, the European rabbit (Esteves et al., 2015).

2.1.1.1.1. RHDV evolution from pre-existing non-pathogenic viruses (NP-LV)

Several authors share the hypothesis that RHDV originated from pre-existing NP-LV that circulated previously in Europe. This hypothesis was put forward after the detection of anti-RHDV antibodies in rabbit blood samples collected long before the first RHDV documented outbreak in 1984 (Moss et al., 2002), and after the discovery and characterization of different NP-LV strains from European rabbits (Capucci et al., 1996)(Moss et al., 2002)(Forrester et al., 2007)(Le Gall-Reculé et al., 2011b). Yet, while some suggested that highly virulent RHDV strains evolved a number of times independently close to 1984 (Moss et al., 2002)(Forrester et al., 2006a)(Forrester et al., 2006b),

others proposed that RHDV originated in a single event in China, from where it spreaded, causing severe epidemics worldwide (Kerr et al., 2009).

2.1.1.1.1.1. Multiple and independent virulent RHDV evolution and emergence

Moss et al., (2002) disclosed the existence of RHDV strains dating back to 1955 in the United Kingdom (UK). Their nucleotide sequences, which clearly predate the first RHD description, were obtained by nested RT–PCR from archived blood samples provided by healthy commercially supplied rabbits (Moss et al., 2002). The phylogenetic studies carried out by Moss et al., (2002) and Forrester et al. (2006a), which included these early 1950s sequences, showed that this sequences diverged from the strains detected in China. In addition, the pathogenic RHDV viruses that emerged in the UK in the early 1990s were also distinct from and pre-dating those emerging in China (Forrester et al., 2006a) (**Figure 20**). In this temporal contradictory scenario, Moss et al. (2002) and Forrester et al. (2006a) hypothesized that RHDV emerged in Europe from mutation of a previously circulating avirulent/non-pathogenic virus, to cause epidemic outbreaks in this geographic area independently of the Chinese epidemic virus (Moss et al., 2002)(Forrester et al., 2006a). According to this hypothesis, virulence probably emerged multiple times independently around 1984, but at least twice, one in Europe and another in China (Forrester et al., 2006a)(Forrester et al., 2006b).

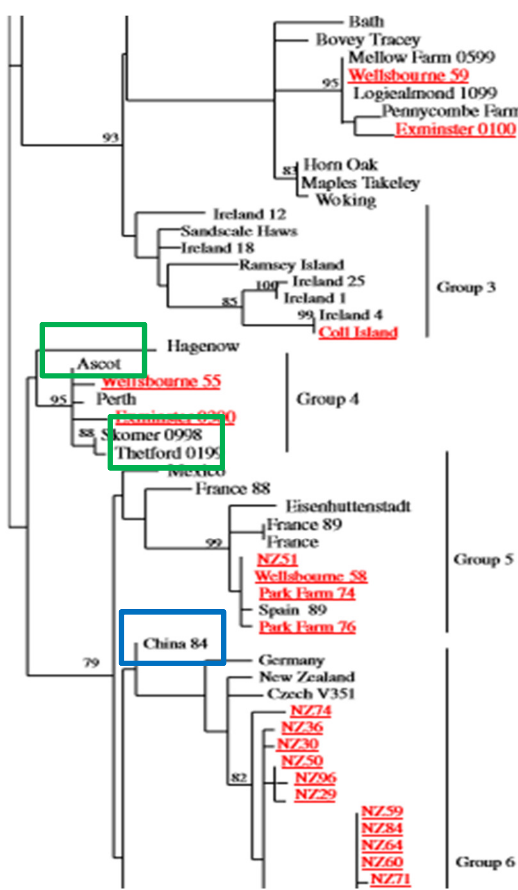


Figure 20. Fraction of a maximum likelihood (ML) phylogenetic analysis performed by Forrester et al. (2006a) using partial RHDV capsid sequences. Viruses isolated from healthy rabbits are underlined and those isolated from rabbits presumed to have died from RHDV are in normal text. The "China 84" strain is marked in blue and two of the pathogenic strains from the UK are marked in green ("Ascot" and "Thetford 0199").

2.1.1.1.1.2. Unique emergence of virulence and virulent RHDV evolution

Kerr et al. (2009) suggested that RHDV originated in China from where it spread to cause severe epidemics. According to the same study, the 1950s strains from UK taken as evidence for the long-standing circulation of RHDV prior to the Chinese outbreak of 1984 (Moss et al., 2002)(Forrester et al., 2006a), corresponded most probably to modern contaminants (**Figure 21**) and, apart from them, no other evidences of the existence of RHDV-*like* viruses, distinct from RCV-*like* viruses, prior the first RHDV outbreak in 1986 were ever elicited in Europe.

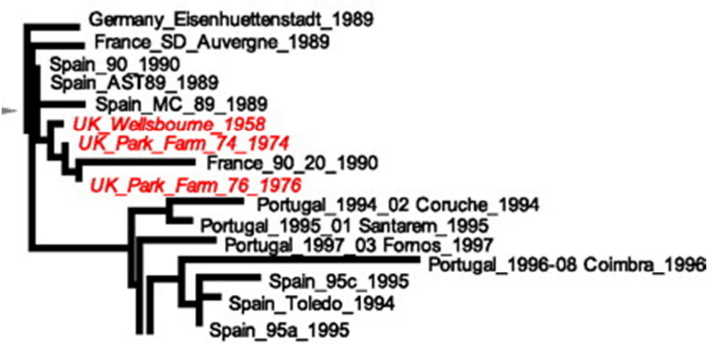


Figure 21. Fraction of a maximum likelihood (ML) ML tree of the RHDV capsid gene performed by Kerr et al. (2009), showing the positions of three of the UK isolates sampled before 1984 (written in red) and their close relationship to more contemporaneous isolates.

Based on the analysis of the complete *vp60* gene, Kerr et al. (2009) estimated a mean evolution rate for RHDV of 7.7x10^{-4} nucleotide substitutions/site/year (ns/s/y) (with 95% highest probability density (HPD) values of 3.9x10^{-4} to 11.9x10^{-4} ns/s/y). These results indicated approximately one substitution per year in the 1740-nt complete capsid sequence (equivalent to two lineages diverging for 0.5 years). Also, NP-LV showed to be clearly divergent from each other and from RHDV with a common ancestor for all viruses dating back to more than 200 years, meaning RHDV did not originate form NP-LV recently. In this context, Kerr et al. (2009) suggested that virulent RHDV strains most probably emerged once, in the early XXth century, but were not detected until 1984, when rabbits' trade provided the opportunity for RHDV to spread from an established, but apparently cryptic, transmission cycle.

For the lack of RHD detection prior to 1984, these authors pointed out two hypotheses. The first, considered that evolution of virulent RHDV took place in Asia in farmed European rabbits as the RHDV emergence in China matched a period of rabbit industrial production expansion. In the complex socio-political context involving China and its neighboring countries in the first half of the XXth century, avirulent/moderatly virulent viruses causing subclinical disease in rabbits could have evolved in this region without leaving records. Moreover, contact with non-pathogenic strains might have provided rabbits some degree of cross-protection against RHDV so that large-scale disease outbreaks may have been uncommon until production intensification. Interestingly, RHD was first described in imported rabbits that may have been immunologically naïve (Liu et al., 1984).

Alternatively, Kerr et al. (2009) raised the less plausible hypothesis where a viral lineage could have

circulated undetected in Europe until 1986 despite spreading to China in 1984. From China, RHDV would have expanded around the world and back in Europe, where it may also have emerged from cryptic foci.

2.1.1.1.2. The species jump hypothesis

Other researchers, such as Esteves et al., 2015, argue that the former theory does not explain the abrupt emergence of highly pathogenic strains on several occasions in such a short period of time. These authors also pointed out that pathogenic and non-pathogenic viruses are phylogenetically distinct, suggesting that the pathogenic viruses did not directly originated from the non-pathogenic ones.

Moreover, several other arguments are in favour of the species jump hypothesis suggesting that RHDV was maintained in a yet unidentified host before it acquired the ability to infect European rabbits. The oldest TMRCA published for RHDV, implies that the coalescent of virulent RHDV antedates the emergence of RHD by almost seven decades. In the absence of an intermediate reservoir host, it is uncommon for the emergence of an acute, virulent virus to be so extensively decoupled from the appearance of its associated disease (Hicks and Duffy, 2012). Furthermore, the previously estimated mean substitution rates for the RHDV *vp60* gene, suggested to range from 5.48×10^{-4} ns/s/y (Alda et al., 2010) to 2.65×10^{-3} ns/s/y (Kinnear and Linde, 2010) with non-overlapping 95% HPD intervals, were recently shown to be higher (Hicks and Duffy, 2012). In fact, after removing from the databases one misdated artefact RHDV taxon, namely a passaged lab strain used for vaccine production, responsible for depressing the RHDV capsid gene's rate of evolution by 65%, the RHDV mean evolution rates based on the polymerase and capsid protein genes were estimated in 1.90×10^{-3} ns/s/y (95% HPD 1.25×10^{-3} to 2.55×10^{-3}) and 1.91×10^{-3} ns/s/y (95% HPD $1.50 \times 10^{-}3$ to 2.34×10^{-3}), respectively (Hicks and Duffy 2012). This refinement placed the RHDV emergence relatively more recent, in 1918 (95% CI: 1893–1941), obviating the need for previously hypothesized decades of unobserved diversification of the virus (Hicks et al., 2012)(Le Gall-Reculé et al., 2013).

In this new scenario, the species jump hypothesis has gained force in explaining RHDV emergence, with the evolution towards virulence having occurred in a species other than the *Oryctolagus* sp. rabbit (Le Gall-Reculé et al., 2013)(Esteves et al., 2015). The discovery of such reservoir host species would be crucial to support this hypothesis while facilitating the understanding of pathogenic lagoviruses emergence in rabbits and perhaps also in hares (Le Gall-Reculé et al., 2013). In the past,

some authors rejected the species jump based on the fact that RCV-*like* and RHDV isolates were closely related (and any virus that had recently jumped species boundaries was likely to have much deeper roots) and that there was no evidence for such a reservoir host (Kerr et al., 2009).

According to Esteves et al. (2015), a likely candidate species from which the species jump might have occurred is the eastern cottontail rabbit (*Sylvilagus floridanus*), native to North America (Gibb, 1990) (Esteves et al., 2015). Massive eastern cottontails from the United States were translocated into Europe by hunters in the 1960s, namely into France (1953 and 1972), Italy (1966), Spain (1980) and Switzerland (1982) (Lavazza et al., 2015a), although these introductions are poorly documented mostly because they were illegal (Esteves et al., 2015). Interestingly, RHDV and EBHSV emerged at around the same time, overlapping the introduction of the eastern cottontail in Europe (Esteves et al., 2015)(Lavazza et al., 2015a).

The phylogenetic study carried out by Kinnear and Linde (2010) had already suggested the possible origin of RHDV and NP-LV among the diverse leporid fauna of North America. These authors estimated the most recent common ancestor (MRCA) of rabbit caliciviruses, including RCV (Capucci et al., 1996), to have existed around 1931 (95% HPD 1897–1961). This falls within the timescale of the development of breeds and husbandry methods for intensive rabbit production, which originated in North America and were introduced into Europe in the 1950s and 1960s (Lebas et al., 1997)(Kinnear and Linde, 2010).

A recent serological study by Lavazza et al., (2015a) on the role of eastern cottontails as a host, vehicle or reservoir of lagoviruses in Italy, showed seroprevalences of 18% and 33% for EBHSV and RHDV antibodies, respectively (these percentages were reduced to 11.9% and 14.3%, respectively, when considering titres equal to or higher than 1/20). However, with regard to RHDV serology, the titres detected were consistently too low to be considered directly induced by RHDV or by a cross-reaction with EBHSV-induced antibodies.

Esteves et al. (2015) hypothesized the original virus would have caused only a benign symptomatic infection in its natural host but lethal in the European rabbit following a species jump. A recent experimental work confirmed that cottontails are not susceptible to RHDV infection (Lavazza et al., 2015a), as previously observed (Gregg et al., 1991), although susceptible to EBHSV with only sporadic cases of clinical disease (Lavazza et al., 2015a). Nevertheless, the prevalence of low titre RHDV-positive sera was higher than that for EBHSV, strongly suggesting the putative presence of a non-pathogenic lagovirus in cottontails genetically related to those found in European rabbits (Capucci et al., 1996)(Lavazza et al., 2015a).

Also supporting the species jump hypothesis is the recent report of cross-species transmission of classical RHDV to Iberian hares (*L. granatensis*) (Lopes et al., 2014). Two different classical RHDV strains were identified in Iberian hares collected dead in 1996 and 1998, respectively, representing two independent infections (Lopes et al., 2014) (**Figure 22**). Until then, RHDV was considered species-specific (Capucci et al., 1996)(Lopes et al., 2014). These evidences, along with RHDV2 capacity to infect the Sardinian Cape hares (*Lepus capiensis mediterraneus*) (Camarda et al., 2014) (**Figure 23**) the Italian hares (*L. corsicanus*) (Puggioni et al., 2014) and the European brown hare (*L. europaeus*) (Lavazza A, *personal communication*) causing RHDV-like disease, are in favour of species jumps of lagoviruses between leporid species (Lopes et al., 2014)(Esteves et al., 2015).

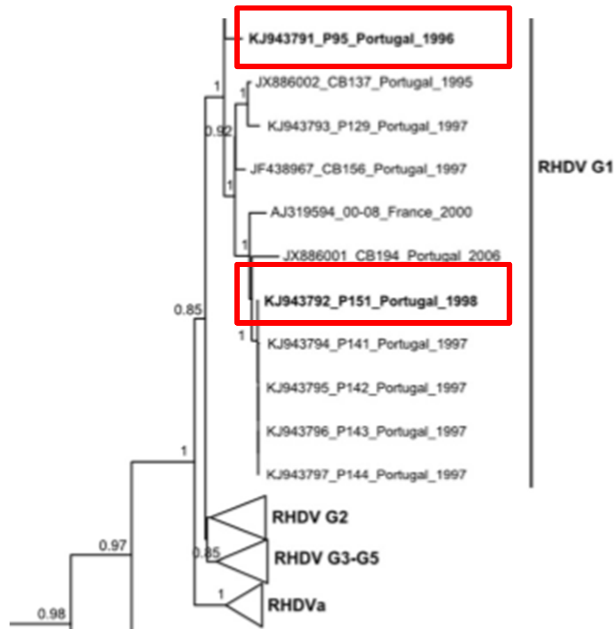


Figure 22. Fraction of the maximum likelihood (ML) tree of the *vp60* capsid gene of lagoviruses performed by Lopes et al. (2014). The RHDV sequences obtained from Iberian hares are marked in red (accession numbers KJ943791 (1996) and KJ943792 (1998)) and group with other RHDV strains from genogroup G1.

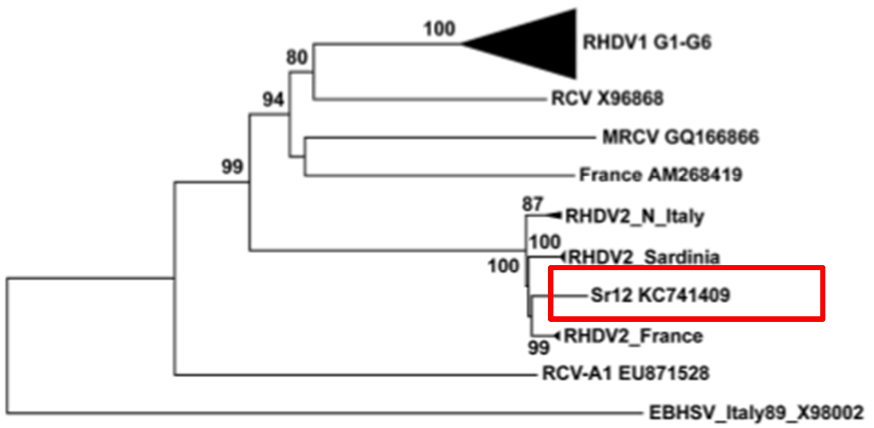


Figure 23. Neighbor-Joining (NJ) tree performed by Camarda et al. (2014), evidencing that the strain Sr12 RHDV2, the RHDV2 strain infecting the Italian hare (accession number KC741409), clustered together with previous identified RHDV2 strains, forming a distinct clade separated from RHDV and RCV.

Esteves et al. (2015) considered that European leporids carry lagoviruses of two distinct origins, the NP-LV, which have evolved with leporids for a long time, and a second group including pathogenic strains that possibly emerged subsequently to species jumps from the *S. floridanus* and evolved in European leporids since. Moreover, according to these authors, pathogenic strains may correspond to pure cottontail viruses or recombinants of cottontail viruses and non-pathogenic viruses of European leporid species.

2.1.1.1.3 Recombination in the basis of virulent RHDV emergence

Besides the high mutation rates of RNA viruses, genetic variation may also arise by other mechanisms such as homologous recombination amongst closely related RNA molecules (Forrester et al., 2008). The rate of recombination seems to be variable and unique to each virus species and while some recombine very frequently, others appear to be constrained, showing little evidence of recombination (Forrester et al., 2008).

McIntosh et al. (2007) suggested that genetic recombination between new and old RHDV could explain the emergence of highly pathogenic RHDV in Europe, with similarities with *vp60* sequences of NP-LV predating 1984. Persistent RHDV infection in immunised laboratory-reared rabbits after virulent RHDV challenge (Gall and Schirrmeier, 2006), along with the discovery of RHDV antibodies in rabbits with no signs of disease implies RHDV circulation amongst healthy rabbits (Forrester et al., 2007), and the cognizance that different but related strains of RHDV circulate and overlap

geographically throughout Europe (Forrester et al., 2006a)(Forrester et al., 2006b) potentiating interactions between genomes and eventual recombination, constitute several arguments that support this hypothesis (Forrester et al., 2008).

In fact, when studying the capsid gene of several RHDV strains, Abrantes et al. (2008) found evidences of recombination among the RHDV Hartmannsdorf strain, which showed different phylogenetic profiles depending on the region of the capsid examined. Forrester et al. (2008) identified frequent recombination events amongst RHDV strains, hence suggesting their key role in this virus the evolution.

According to Forrester et al. (2008), most caliciviruses present three ORFs, with the non-structural region and the structural region as two separate ORFs. It is more likely for recombination to occur between the non-structural and structural encoding genes. This is not the case for RHDV, which does not show this ORFs division (Meyers et al., 1991b)(Parra et al., 1993). However, these researchers identified two single crossovers in two RHDV genomes at the homologous point where the ORF1 and ORF2 occur in other caliciviruses. This point corresponds to the cleavage site of the capsid gene during post-translational processing.

Other recombinant RHDV strains showed evidence of a small insertion, involving a small region of ≈500 bp from one virus genome into another (Abrantes et al., 2008)(Forrester et al., 2008). This insertion, located in the region of the p37/29 cleavage point lengths the cleavage point itself, possibly requiring high conservation in this region to facilitate the protease activity. Hence, recombination may be more prevalent here as sequences are conserved. These small genetic insertions currently represent a difference between RHDV and other caliciviruses (Forrester et al., 2008).

The phylogenetic study carried out by Kerr et al. (2009) argued against large-scale recombination, by showing generally congruent groups with a clear geographic structure. Recombination was considered a rare phenomenon, with little effect on estimating of time spans to common ancestry as determined by Kerr et al. (2009), who excluded it as a viable explanation for RHDV origin.

2.1.1.2 RHDV genetic diversity

RHDV strains are classified based on their complete capsid amino acid sequences (Kinnear and Linde, 2010). Although RHDV is a RNA virus, prone to mutations, the RNA and protein sequences of different RHDV isolates is highly conserved (Nowotny et al., 1997)(Le Gall et al., 1998)(Asgari et al., 1999)(Le Gall-Reculé et al., 2003). Nevertheless, despite this low level of genetic variation, molecular

characterization of RHDV strains allowed the distinction of six well-defined phylogenetic genogroups (G1 to G6) (**Figure 24**) (Nowotny et al., 1997)(Le Gall et al., 1998). There is a single RHDV serotype divided in two subtypes: the classic RHDV genogroups (G1 to G5) and the antigenic variant RHDVa (or G6) (Le Gall-Reculé et al., 2003). Nevertheless, cross-protection with G1-G6 is almost complete (Le Gall-Reculé et al., 2013).

VP60 capsid protein sequences from RHDV strains, representing the six genogroups, revealed seven domains (V1 to V7) in which the highest amino acid variability is observed (Wang et al., 2013). The maximum nucleotide divergence was found between isolates from genogroups G5 and G6 (up to 9.9%), despite the fact that they were isolated during the same period (Le Gall-Reculé et al., 2003). In NP-LVs the variability exceeds these seven domains, encompassing the complete capsid protein (Le Gall-Recule et al., 2013).

Although some studies suggested that RHDV genogroups link to variables such as the years of sampling (Nowotny et al., 1997)(Le Gall-Reculé et al., 1998)(Kerr et al., 2009) (Le Gall-Reculé et al., 2003), geographic origin (Alda et al., 2010), or virulence of strains, these relations were not always observed and therefore cannot be generalized (Müller et al., 2009).

The emergence of the antigenic variant or subtype G6 was of particular interest, and it will be addressed with more detail before the RHDV phylogeography description.

2.1.1.2.1 The antigenic variant G6/RHDVa

The G6 genogroup forms a distinct genetic group (Le Gall-Reculé et al., 2003) and was identified for the first time in 1996, both in Italy (Capucci et al., 1998) and Germany (Schirrmeier et al., 1999), before spreading throughout the world causing epidemics (Grazioli et al., 2000)(Farnós et al., 2007) Le Gall-Reculé et al., 2003)(McIntosh et al., 2007)(Tian et al., 2007)(Kerr et al., 2009).

However, some researchers suggested that related viruses were already present in China as early as 1985 (Kerr et al., 2009). In fact, G6 was shown to have diverged prior to 1984, having relatively deep roots and a MRCA around 1966 (Kerr et al., 2009)(Kinnear and Linde, 2010). This suggests that G6 did not evolved recently, as previously proposed by McIntosh et al. (2007).

Kinnear and Linde (2010) proposed the role of a rapid antigenic selection in the evolution of RHDV, promoting variation in evolutionary rates between nucleotide sites and between lineages. Hence, a selective advantage was suggested for G6 infectivity or replication over the original RHDV serotype (McIntosh et al., 2007). Supporting this hypothesis is fact the G6 formed a distinct phylogenetic clade, showing the highest relative genetic diversity within RHDV and hence the highest effective

population size (Kerr et al., 2009).

G6 replaced the classical strains in some countries, namely in Italy (Grazioli et al., 2000), France (Le Gall-Reculé et al., 2003), and Hungary (Matiz et al., 2006) and in wild rabbits from the Netherlands, where it was related with the decline of *O. cuniculus* (van de Bildt et al., 2006)

Figure 24. Phylogenetic tree derived by the neighbor-joining (NJ) method (Le Gall-Reculé et al., 2003) showing that RHDV sequences are clustered into six major genogroups (G1 to G6).

2.1.1.2.2 RHDV phylogeography

2.1.1.2.2.1. RHDV genetic diversity in France

In France, RHDV isolates were assigned into 5 genetic groups (G1 to G5) showing a temporal distribution (Nowotny et al., 1997)(Le Gall et al., 1998)(Le Gall-Reculé et al., 2003). Most genogroups were, however, successively replaced (Le Gall-Reculé et al., 2003). Genogroups G1 and G2 congregated isolates collected between 1987 and 1990, and were both present simoultaneously (Le Gall-Reculé et al., 2003). They were subsequently replaced by G3 viruses (Le Gall et al., 1998). Genogroup G4 is thought to have emerged from genogroup G3, which then disappeared, as these genogroups were not supported by high bootstap values and most G4 mutation hot spots were identical to those found in genogroups G1 and G3. On the other hand, genogroup G5 was considered an independent group with distinct mutational hot spots (Le Gall-Reculé et al., 2003). This could explain why genogroups G4 and G5 co-existed between 1994 and 1999 (Le Gall-Reculé et al., 2003). From 1999 onwards, genogroup G4 disappeared with all strains clustering into genogoup G5, which included the most recent isolates from mainland France (Le Gall-Reculé et al., 2003) and corresponded to the main genetic group circulating in France until the emergence of RHDV2 (Le Gall-Reculé et al., 2013).

2.1.1.2.2.2. RHDV genetic diversity in Australia

The RHDV evolution over a 16 year period (1995–2011) was evaluated by Kovaliski et al. (2014) by VP60 sequences phylogenetic analysis. The Australian viruses form a monophyletic group with the inoculum strains, the Czech CAPM V-351 and RHDV351INOC, falling near the root of this cluster (**Figure 25**). The CAPM V-351 strain was used (via passage) to manufacture the suspension RHDV351INOC for laboratory trials and inoculation of field rabbits. This phylogenetic pattern indicates a single introduction of RHDV in Australia from these two inoculum strains and that, despite several reintroductions of RHDV351INOC strain to maximise the impact of the disease, only a single viral lineage (*i.e.* that derived from the initial release) became established and sustained its long-term transmission, suggesting its major competitive advantage. The close evolutionary relationship between the Australian and New Zealand viruses indicates a common virus source in both countries.

It is possible that the fitness of the founding lineage may have been enhanced due to a largely susceptible rabbit population. However, this main lineage has also experienced a major 'turnover'

throughout time, as viral lineages replaced each other in specific geographical localities, particularly in New South Wales and South Australia. Evidences of widespread viral gene flow were obtained, in which multiple lineages entered individual geographic locations, resulting in a marked turnover of viral lineages with time and a continual increase of genetic diversity. Despite the RHDV geographical clustering by state of sampling was observed, the structure of RHDV genetic diversity changed with time as multiple lineages entered certain regions, namely Australian Capital Territory, New South Wales and South Australia. As an example, RHDV strains from Australian Capital Territory from 2009 onwards fell into two lineages, both of which were distinct from a more basal lineage from 1998, indicating a lineage replacement. This lineage turnover suggests that viruses do not persist at individual geographic localities between disease outbreaks but are possibly imported each year by insect vectors such as blowflies into rabbit populations (Asgari et al., 1998).

The mean rate of RHDV evolution recorded in Australia ranged from from 4.0 – 4.7 × 10^{-3} ns/s/y (Kovalinski et al., 2014), substantially higher than all previous rate estimates for this virus (Kerr et al., 2009)(Kinnear and Linde, 2010) and one of the highest observed in RNA viruses (Kovalinski et al., 2014).

The TMRCA for the Australian epidemic estimated (1994.4 – 1995.4) was in accordance with the date of the initial release of the virus in the country and there was a clear association between genetic distance and date of sampling (correlation coefficient = 0.85) indicating that there is strong temporal (*i.e.* molecular clock) structure in the data. An exponential increase in RHDV genetic diversity across time can be interpreted as a concomitant increase in viral population size under a neutral evolutionary model. Such population growth suggests that ecological, genetic, and immunological constraints, including the co-circulation of the benign RCV-A1 or rabbit resistance, did not impose a major selective challenge to RHDV (Kovalinski et al., 2014).

In brief, the RHDV phylogenetic pattern observed in Australia is consistent with a single introduction of RHDV into the country from the inoculum strains, followed by *in situ* evolution over the next 16 years (Kovalinski et al., 2014).

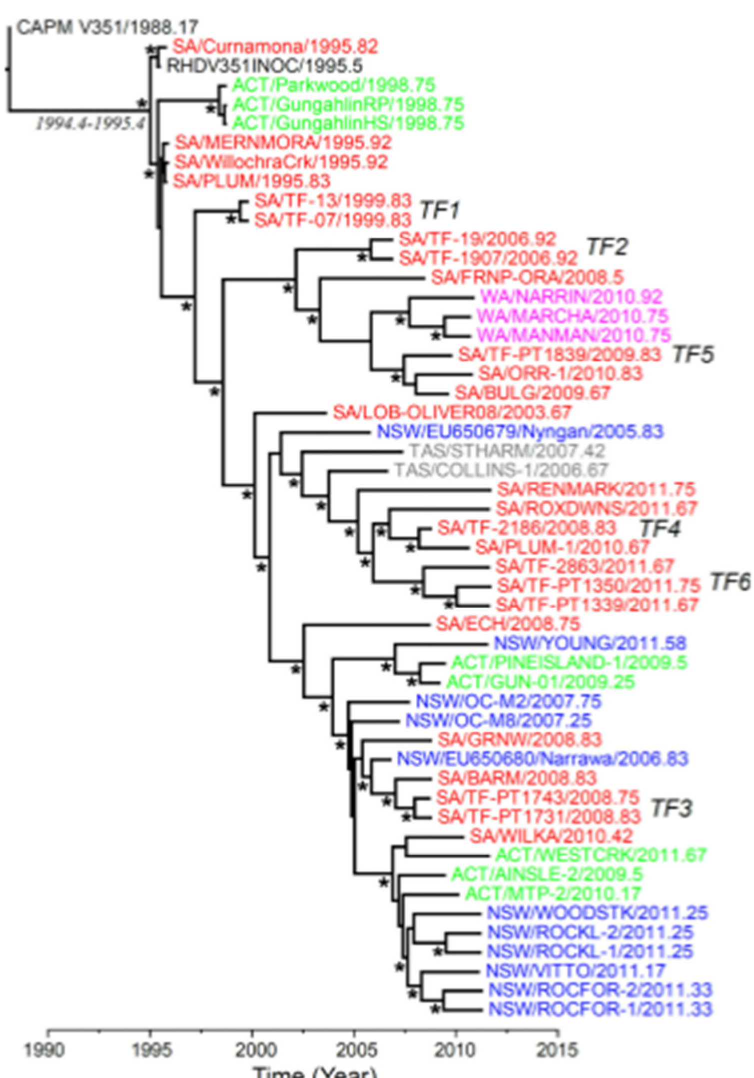


Figure 25. Phylogenetic tree derived by the maximum clade credibility (MCC) method performed by Kovaliski et al. (2014) depicting the evolutionary relationships among the Australian RHDV sequences, forming a monophyletic group with the inoculum strains Czech CAPM V-351.

2.1.1.2.2.3. RHDV genetic diversity in the Iberian Peninsula

In the Iberian Peninsula, RHDV was first detected in 1988 in Spain (Argüello-Villares et al., 1988) and in 1989 in Portugal (reviewed by (Duarte et al., 2014)). However, its origins remain to be clarified regarding whether RHDV was already circulating in an avirulent form, or of if it originated from a single or multiple introductions (Alda et al., 2010). The phylogenetic study carried out by Alda et al. (2010) suggested that all the Iberian RHDV field strains have a common ancestor and are closely related to strains isolated during the first RHD outbreaks in Spain (AST/89 and MC-89). The age of the MRCA of the Iberian strains was the youngest (~1936, 95% HPD: 1955.7-1869.2) of all the regions analysed by the authors, yet predating the first RHD outbreak in 1984, in China.

The phylogenetic study carried out by Müller et al. (2009) using samples originating from Spain, Portugal and South of France collected between 1994 and 2006 showed that these sequences clustered into three groups designated the "Iberian" (IB) groups IB1, IB2 and IB3. Together with G1 sequences, these three IB groups formed a distinct cluster separated from all RHDV genogroups previously described. Groups IB2 and IB3 shared a common ancestor with genogroup G1 viruses but group IB1 (containing five sequences from central Portugal) separated before genogroup G1. Three additional Iberian clades were later described by Alda et al. (2010): IB4, IB5, IB6. No clear geographic structure was observed among the Iberian samples analysed by these researchers, although most of the clades were restricted in time, with the exception of IB3, the most widespread clade both in time and space.

None of the RHDV sequences from Spain and Portugal clustered within genogroup G2 suggesting that only G1-related strains predominated initially and were subsequently replaced by IB2 and IB3 strains, indicating that RHDV could have evolved separately in the Iberian Peninsula since then (Müller et al., 2009). Müller et al. (2009) found that the nucleotide substitutions observed in groups IB2 and IB3 seem to have become fixed around 1994 and were still present in the RHDV strains circulating in wild rabbits in the Iberia to the date of the study (2009), but not elsewhere. These findings suggested evidences of genetic isolation of the Iberian Peninsula starins, with the Pyrenees acting as a major natural barrier, constraining wild rabbit and hence viral dispersal and evolution (Müller et al., 2009)(Alda et al., 2010). In fact, genogroup G1 was able to persist only in the Iberia (Lopes et al., 2014).

The presence of G6 on the Iberian Peninsula, where this variant was thought not to contribute to viral diversity, was also reported but in sporadic cases in farms and never in wild populations

(Müller et al., 2009)(Abrantes et al., 2014)(Duarte et al., 2014)(Lopes et al., 2014).

2.1.1.2.2.3.1 RHDV genetic diversity in Portugal mainland

All RHDV strains circulating in Portugal mainland identified by the phylogenetic study carried out by Müller et al. (2009) were G1-related. These strains, collected between 1994 and 2006 fell into two distinct groups, namely IB1 and IB2 (**Figure 26**). Within temporal groups, a link to its place of origin was also observed suggesting that two RHDVs were circulating concomitantly in the presence of disease and mortality. Supporting this hypothesis, the same authors found that IB1 strains differed in eight positions from IB2 strains (although only in three when compared to G1).

The antigenic variant G6 was also identified in a commercial rabbitry in the North of Portugal (Müller et al., 2009). The retrospective phylogenetic study carried out by Duarte et al. (2014) corroborate these results by disclosing that RHDV strains circulating in Portugal mainland fell within genogroups G1 and G6. G1-related strains belonging to the IB3, and G6 were identified among samples collected in Portugal mainland between 2006 and 2008 (**Figure 26**), demonstrating the circulation of these strains until at least 2008 (Duarte et al., 2014). Recently, RHDV G1-related strains were also detected in two Iberian hares found dead in the field in Portugal in 1996 and 1998 (Lopes et al., 2014).

Figure 26. Fraction of the phylogenetic tree derived by the neighbor-joining (NJ) method based on the analysis of the partial vp60 gene from RHDV performed by Müller et al. (2009) depicting that the RHDV strains from Portugal clustered separately from known genogroups and showing the Iberian groups (IB) 1, 2 and 3.

2.1.1.2.2.3.2. RHDV genetic diversity in the Azorean archipelago

In the Azores, the first RHDV outbreaks were recorded from 1988 to 1993 (Carvalho and Almeida, 1991)(Martins,1993)(Carvalho et al., 1993)(Carvalho et al., 1994) but only in 2014 the circulating strains were characterized (Esteves et al., 2014)(Duarte et al., 2014).

The retrospective phylogenetic study carried out by Duarte et al. (2014) disclosed that G5 strains were the dominant group in the Azorean islands. This genogroup was never identified in Portugal mainland (Duarte et al., 2014).

RHDV collected in 2013 from Azores revealed unique characteristics and formed a highly

supported group that, although more closely related with the RHDV strains G1–G5, they were distinct differing from each RHDV groups (G1, G2 and G3–G5) by approximately 8% (between group means distances) (Esteves et al., 2014). Evidences for recombination were not found (Esteves et al., 2014). In this scenario, all three main groups (G1, G2 and G3–G5) could have been at the origin of the RHDV strains detected in Pico Island. These evidences suggested an independent RHDV evolution in Azores after an initial introduction more than 17 years ago (Esteves et al., 2014).

2.1.2. RHDV2

2.1.2.1 RHDV2 origin and evolution

RHDV2 is a new virus highly distinct from the pathogenic and non-pathogenic lagoviruses described before its emergence (Le Gall-Reculé et al., 2013). RHDV2 has unique features and quickly dispersed from France, where it was first reported in 2010 (Le Gall-Reculé et al., 2011a).

Presently the virus circulates in several European countries, namely in France (Le Gall-Reculé et al., 2011a)(Le Gall-Reculé et al., 2013), Italy (Le Gall-Recule et al., 2013), Spain (Dalton et al., 2012), Germany (information on the FLI, 10|21|2013), Portugal mainland (Abrantes et al., 2013) and archipelago of Azores (Duarte et al., 2015b), England and Wales (Westcott et al., 2014), Scotland (Baily et al., 2014) and Finland (http://www.oie.int/wahis_2/public/wahid.php/Countryinformation/Countryreports). RHDV2 was also detected in Tenerife (Canary Islands) (Martín-Alonso et al., 2015) and in Australia (Hall et al., 2015). Tunisia, the Scandinavian countries of Norway (OIE Technical disease cards. 2016) and Sweden (Neimanis et al., 2017), the Ivory Coast (http://outbreakwatch.blogspot.pt/2016/09/proah-rabbit-hemorrhagicdisease-cote.html) and Canada (http://outbreakwatch.blogspot.pt/2016/08/proahedr-rabbit-hemorrhagic-disease_26.html) reported the disease in 2016.

Despite the virus presence was only reported by Sweden in 2016, the Neimanis et al., (2016) study suggests that the virus was already present in the country as early as May of 2013 and is currently the dominant cause of RHD in the country.

Recently, by the end of 2016, the virus was also detected by our team in the Madeira archipelago (Carvalho et al., 2017c).

Similarly to what happened for classical RHDV emergence, two possible hypothesis have been put forward to explain RHDV2 emergence, the evolution from a pre-existing non-pathogenic virus or a species jump from a reservoir host species (Le Gall-Reculé et al., 2013)(Esteves et al., 2015).

However, molecular data seem to suggest that RHDV2 did not emerge following the genetic evolution of previously known lagoviruses, despite this hypothesis cannot be definitively excluded as knowledge of NP-LVs is yet scarce (Le Gall-Reculé et al., 2013).

2.1.2.1.1. RHDV2 genetic diversity

Phylogenetic studies on RHDV2 have shown that all sequences cluster together and separatly from RHDV strains (**Figure 27**). This new virus showed to be more closely related to RHDV and RCV-like viruses than to the independent RCV-A1 genetic group (Le Gall-Reculé et al., 2011a)(Le Gall-Réculé et al., 2013). The average nucleotide identity between RHDV2 and G1 to G5, and G6 obtained by Le Gall-Reculé et al. (2013) ranged from 82.4% to 85.7%, respectively. These values are in agreement with the data later obtained by Lopes et al. (2014), which showed that RHDV2 presented an overall identity of 82% with G1 strains, 81.6% (± 0.8%) with G2, 82.2% (± 0.8%) with G3-G5, and 82.1% (± 0.8%) with G6. Regarding the identity with NP-LVs, on average RHDV2 shared 81% (± 1.0%) identity with RCV, 06–11 and MRCV strains, and 79.2% identity with RCV-A1 isolates (Le Gall-Reculé et al., 2013)(Lopes et al., 2015a).

Most of the substitutions were located in the most variable part of the capsid protein, namely in the C-terminal region of VP60 (Le Gall-Reculé et al., 2013). When the P domain, displaying the highest degree of genetic variation (Wang et al., 2013), of RHDV2 and NP-LV VP1 were compared, similarities consistently decreased (RCV-A1 (65.3%), RCV (63.2%), RHDV (60%) MRCV (59.2%) 06–11 (53%)), emphasising significant differences between the viruses (Le Gall-Reculé et al., 2013).

When RHDV2 and G6 sequences were compared within the same variable regions defined by Wang et al. (2013), similarities between both viruses also decreased to 60% (Bárcena et al., 2015).

RHDV2 showed to be more closely related to rabbit lagoviruses than to EBHSV, with which it presented only 70.0% (± 0.9%) nucleotide identity (Le Gall-Reculé et al., 2013)(Lopes et al., 2015a).

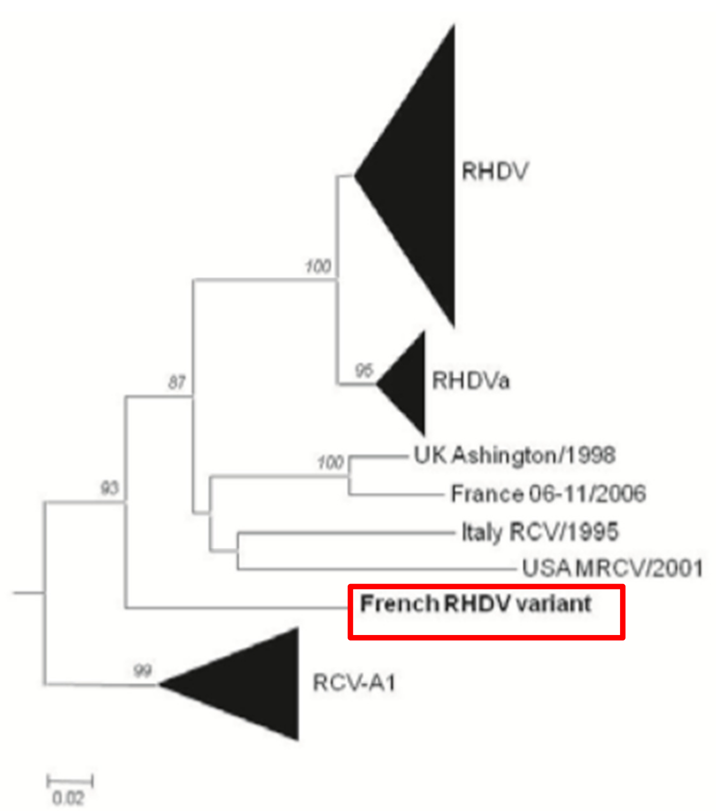


Figure 27. Phylogenetic tree by Le Gall-Reculé (2011) using the minimum evolution method derived from partial partial *vp60* sequences of lagoviruses showing that RHDV2 (French RHDV variant) forms a new genetic group, distant from the pathogenic and NP-LV lagoviruses.

2.1.2.1.1.1. RHDV2 genetic diversity in the Iberian Peninsula

In the Iberian peninsula RHDV2 was first detected in Spain in 2011 (Dalton et al., 2012). In Portugal the virus was identified in the following year (Abrantes et al., 2013). All Iberian strains appear to have derived from a common ancestor shared also by a unique non-Iberian strain collected from a *Lepus corsicanus* specimen in Italy mainland (Duarte et al., 2015). Strains from Portugal share a common ancestor (Duarte et al., 2015). The current circulating strains in mainland Portugal seem to have resulted from multiple recombination events between RHDV2 and non-pathogenic or pathogenic G1 strains, with a single breakpoint located in the 5′ region of VP60 (Lopes et al., 2015b). This breakpoint divides the genome in two regions, namely one encoding the non-structural proteins, and another encoding the major (VP60) and minor (VP10) structural proteins (Lopes et al., 2015b). Furthermore, two types of recombinants with distinct genomic background were found, including the RHDV2 structural proteins with non-structural proteins from non-pathogenic lagoviruses (NP-LV) or from G1 (Lopes et al., 2015b).

The phylogenetic analysis based on *vp60* and *vp10* genes showed that all Iberian RHDV2 viruses

clustered together and apart from the other strains (**Figure 28**), but for non-structural protein encoding genes these strains clustered in three different groups (Lopes et al., 2015).

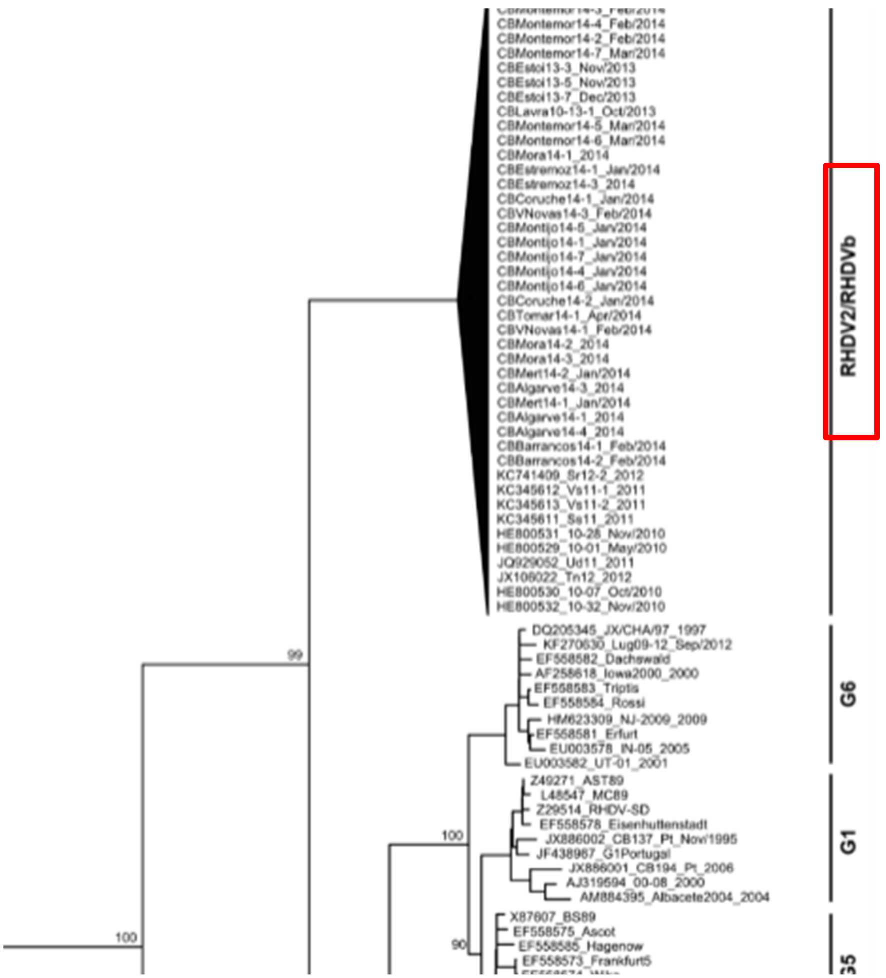


Figure 28. Fraction of the maximum likelihood (ML) phylogenetic tree of the *vp60* gene of lagoviruses performed by Lopes et al., (2015) showing that the RHDV2 sequences collected in Portugal mainland clustered together with high bootstraps and apart from other lagoviruses, namely RHDV.

2.1.2.1.1.2. RHDV2 genetic diversity in the Azorean archipelago

In the Azorean archipelagos, RHDV2 was first detected in 2015 in wild rabbits from Graciosa, Flores São Jorge and Terceira islands (Duarte et al., 2015b) and later in Faial, Santa Maria and S. Miguel islands (Duarte et al., 2015a).

Nucleotide analysis showed that the Azorean strains are closely related to each other, sharing a high genetic identity (>99.15%). Despite the high nucleotide identities between the Azorean strains and the ones from Portugal mainland and Spain, no absolute similarity was found between any

strain from Azores and RHDV2 sequences from other geographic origins (Duarte et al., 2015a). A phylogenetic analysis carried ou during this work (**Study 8** of this chapter) showed that the Azorean-RHDV2 formed a strongly supported monophyletic group which clustered separately within the European-RHDV2 group apart from the remaining strains from continental Portugal (Duarte et al., 2015a).

The independent clustering and the low genetic distances found between the Azorean strains, is in favour of a unique RHDV2 introduction in these islands, during late 2014 and suggests an epidemiological link (Duarte et al., 2015a). The initial introduction seems to have occurred in Graciosa, where the first dead rabbits were detected, followed by a rapid expansion to other islands (Almeida et al., 2015). Indeed, the Almeida et al., (2015) study showed that the highest haplotype diversity was found in Graciosa and a star-like topology was associated with the most ''central'' Graciosa haplotype, consistent with a rapid viral expansion. Also, viral sequences from Faial and São Jorge showed to be identical to sequences from Graciosa, suggesting the virus introduction from Graciosa or with an identical strain. However, the RHDV2 sequences from S. Jorge appeared in different clusters, although closely related to sequences from Graciosa, suggesting multiple introductions to S. Jorge from Graciosa might have occurred. Dispersion to S. Miguel seems to have occurred from Terceira.

As the dispersal route did not always related with geographic proximity of the islands, some hypothesis were put forward to explain virus dissemination in the archipelagos, such as the involvement of insects (flies and mosquitoes) considered important vectors for RHDV (McColl et al., 2002a), birds or rodents (Almeida et al., 2015)(Duarte et al., 2015a). However, considering the chronology of the outbreaks, the distance and the frequent maritime traffic between the islands, and the most probable man-mediated RHDV2 arrival to Azores, it seems plausible that RHDV2 spread between islands was also related with anthropogenic movements (Almeida et al., 2015) (Duarte et al., 2015a).

The RHDV2 strains circulating in Azores were shown to be recombinants between G1 and RHDV2, as while the highest homology in VP60 sequences was found with Iberian RHDV2 strains, the upstream fragment revealed high similarity (≈95%) with Iberian G1 strains (Almeida et al., 2015). Considering that G1 was shown to persist only in the Iberian rabbit populations and was successfully replaced by RHDV2 strains currently circulating in the Iberian Peninsula (Lopes et al., 2015a), this data suggested that the RHDV2 strains circulating in Azores most probably originated in Iberian strains (Almeida et al., 2015).

2.1.2.1.1.3. RHDV2 genetic diversity in the Madeira archipelago

Recently we carried out a phylogenetic investigation (**Study 9** of this chapter) showing that the *vp60* sequences from the Madeira archipelago, including The Porto Santo and Madeira islands, grouped together with high bootstraps. Five haplotypes were identified and an overall similarity of 99.54% to 99.89% was observed between the two viral populations.

Seven single nucleotide polymorphisms (SNPs) were identified in the Madeira archipelago 2016-2017 strains, of which two were non-synonymous encoding an Asn_{480} or a Ser_{480} and a Glu_{570}. None of these amino acids have been detected before in this position, given that a Thr_{480}, and less frequently an Ala_{480}, are usually found. Similarly, in position 570 only a Gly_{570} had been identified to date. Additionally, six synonymous and two non-synonymous (encoding residues 347 and 369) SNPs were also found in the viruses from the Madeira archipelago, only shared by few other strains. Altogether, the seven specific and eight rare SNPs defined a DNA fingerprint for the RHDV2 2016-2017 strains from the archipelago (Carvalho et al., 2017c).

2.1.2.1.2 RHDV2 phylogeography

From 2011 onwards, RHDV2 almost completely replaced the circulating classical RHDV strains in France (Le Gall-Reculé et al., 2013), in the Iberian Peninsula (Lopes et al., 2015a) and Azores islands (Duarte et al., 2015a) as well as in Sweden (Neimanis et al., 2016). The factors contributing to this rapid takeover are yet to be clarified but RHDV2 high transmission rate may suggest a selective advantage of this virus over classical RHDV, probably by overcoming the existing immunity induced by the classic strains (Lopes et al., 2015a).

However, despite in Sardinia Island several RHDV2 cases were registered in a very short period indicating the rapid RHDV2 dissemination, in Italy RHDV2 did not spread within continental land and the replacement of circulating RHDV or G6 isolates, responsible for most outbreaks in small rural units, has not been observed (Le Gall-Reculé et al., 2013). This is thought to be related to the widespread presence of wild rabbits in Sardinia, whereas in continental Italy they are sparse and patchily distributed (Fouchet et al., 2009)(Le Gall-Reculé et al., 2013). RHDV2 selective advantage migth be only expressed where wild populations of rabbits are present, similarly to what was observed for RHDV, highly dependent on wild rabbits' densities, indicating that wild populations may exert strong selection pressure on the virus (Le Gall-Reculé et al., 2013).

2.2. Non- and moderately pathogenic lagoviruses (NP-LV)

The intensified surveillance on RHDV resulted in the identification of NP-LVs (Hicks and Duffy 2012). Retrospective serologic studies evidenced the presence of RHDV antibodies in rabbit populations from Europe (Rodák et al., 1990)(Moss et al., 2002), Australia and New Zealand (Strive et al., 2009) 12 years before the first RHDV outbreak was recognised and reported (Rodák et al., 1990)(Moss et al. 2002). These RHDV antibodies were putatively attributed to the contact with non-pathogenic caliciviruses antigenically related to RHDV (Rodák et al., 1990)(Moss et al., 2002).

The existence of non-pathogenic RHDV strains was first documented in 1996 in Italy, after the characterization of a non-lethal RHDV-like strain designated rabbit calicivirus (RCV) (Cappucci et al., 1996). In the subsequent years, other non-pathogenic and moderately pathogenic viruses were also identified in Europe (Moss et al., 2002)(Forrester et al., 2007)(Le Gall-Reculé et al., 2011b), Australia (Strive et al., 2009) and North America/USA (Bergin et al., 2009) in domestic or/and wild rabbits, highlighting the extent of diversity within the *Lagovirus* genus (Le Gall-Reculé et al., 2011b). These included the Ashington strain from the United Kingdom (Moss et al., 2002), the Lambay strain from Ireland (Forrester et al., 2007), the rabbit caliciviruses Australia 1 (RCV-A1) (Strive et al., 2009), the Michigan rabbit calicivirus (MRCV) from North America (Bergin et al., 2009) and the 06-11 strain detected in France more recently (Le Gall-Reculé et al., 2011b).

For these viruses, Kerr et al. (2009) proposed the designation "rabbit calicivirus-like", to distinguish them from RHDV, and later Jahnke et al. (2010) suggested the same term, "RCV-like", due to their closer phylogenetic proximity with RCV. However, RCV and similar strains did not form a monophyletic group with the RCV-A1 strains from Australia (Jahnke, et al., 2010). Le Gall-Reculé et al. (2011b) proposed the name "non-pathogenic lagovirus (NP-LV)" to designate them.

The non-lethality of RCV, RCV-A1, MRCV and 06-11 strain was experimentally confirmed (Capucci et al., 1996)(Strive et al., 2010)(Le Gall Reculé et al., 2011b) but only assumed for all the other strains, since they were isolated from healthy non-vaccinated rabbits (Le Gall-Réculé et al., 2011).

Experimental studies have proven the existence of a gradient of cross-protection between these non-pathogenic strains and RHDV (Le Gall-Reculé et al., 2011b) from non-protective, irrespective of the titre (strain 06–11) (Le Gall-Reculé et al., 2011b), and partially protective (RCV-A1) (Strive et al., 2010) to fully protective (RCV) (Capucci et al., 1996). These differences, as well as competition between non-pathogenic and pathogenic strains, may explain the variable impact of RHD on rabbit populations (Le Gall-Reculé et al., 2011b).

In the Iberian Peninsula, the drastic reduction in wild rabbit numbers caused by RHDV was

historically unprecedented, suggesting that NP-LV were either not present or at a very low prevalence and, if so, highly host-adapted but not cross-protective (Müller et al., 2009).

The NP-LVs form distinctive genetic groups rather than a monophyletic group (Strive et al., 2009)(Jahnke et al., 2010). The RCV, the Lambay strain and the Ashington strain were shown to form a phylogenetic cluster distinct from the RCV-A1 strains and both these groups separate from the RHDV branch (Strive et al., 2009)(Le Gall-Reculé et al., 2011b). The non-pathogenic European caliciviruses were shown to be more closely related to RHDV than to RCV-A1. RCV-A1 are also more distantly related to RHDV (Jahnke et al., 2010). In NP-LVs the variability exceeds the mentioned seven domains, encompassing the complete capsid protein (Le Gall-Recule et al., 2013).

The mean estimated TMRCAs for most NP-LV was placed between the XIXth or early XXth centuries, coincident with the history of the European rabbit (Kerr et al., 2009). Some authors' suggest that NP-LV spread to Australia and New Zealand in wild and/or domestic rabbits introduced from the United Kingdom. Presumably, similar viruses were introduced with European rabbits to other countries, but these have not been identified due to lack of surveillance (Kerr et al., 2009).

The existence of NP-LV and the evidence of recombination between strains (Abrantes et al., 2008) (Forrester et al., 2008) emphasize that the interaction between pathogenic and non-pathogenic strains are probably more complex than previously assumed (Le Gall-Reculé et al., 2011b).

2.2.1. RCV, Italy

RCV, detected in Italy, showed to be genetically distinct from RHDV, presenting distinct capsid protein encoding sequences, also differing from RHDV in viral load and tissue tropism (Cappucci et al., 1996). The primary sequence of the capsid protein was shown to be more closely related to RHDV than to EBHSV (Capucci et al., 1996). It was hypothesized by Capucci et al. (1996), Lavazza and Capucci (2008) and later by Le Gall-Reculé et al., (2011), when referring to a different NP-LV, that the differences found between RCV and RHDV capsid proteins could explain their different tropism and pathogenicity.

RCV infected rabbits appeared healthy and presented no histopathological lesions, but the virus was shown to confer complete cross-protection to RHDV infection (Capucci et al., 1996). Although RCV widespread in nature is yet to be understood, the results obtained by Capucci et al. (1996) suggest that infeccion by RCV may confer a selective advantage to rabbits exposed to RHDV.

2.2.2 Ashigton strain, UK

In the United Kingdom, a putatively pathogenic virus was identified by Moss et al. (2002) in a rabbit that died with typical RHDV symptoms, assuming death to be related to an infection that also victimized 90% of the local rabbits. However the pathogenicity of the Ashington virus was never experimentally demonstrated. The Ashington strain was found to be genetically distinct from all other viruses including RCV, differing by up to 19% nucleotide and 18% amino acid identity. The highly divergent RCV and Ashington strain must have diverged from the remaining groups more than 40 years ago and probably hundreds or even thousands of years ago.

2.2.3. Lambay Island strain, Ireland

Another apparently non-pathogenic virus was detected by Forrester et al. (2007) in healthy wild rabbits on the Lambay Island, where disease due to RHDV had never been observed. ELISA antibody tests showed detectable RHDV antibodies confirming that a RHDV-like virus circulated amongst the Lambay Island rabbits without causing disease but this strain pathogenicity was not tested experimentally.

Forrester et al. (2007) raised the hypothesis that this virus could be as RCV infectious and contagious but without little or no virulence, and that under appropriate circumstances (as stress), epidemic RHD outbreaks could arise.

In their phylogenetic study, Forrester et al. (2007) showed that the Lambay strain diverged from the Ashington strain (84.9% nucleotide identity) and RCV (81.0% nucleotide identity). The Ashington and Lambay Island lineages emerged after divergence from RCV, possibly separating when the viruses were introduced in England and Ireland, respectively.

The virulent strains from Ireland, identified in 1995 (Collery et al., 1995), were closely related to other European strains, having emerged more recently than the Lambay lineage. According to Forrester et al. (2007), the Lambay strain might have circulated within the Irish rabbit population being however outcompeted by the more recently introduced strain(s) and only enduring in isolated pockets.

2.2.4. Rabbit calicivirus Australia 1 (RCV-A1), Australia

Antibodies that cross-reacted in RHDV specific ELISAs were detected in Australian wild rabbits prior to RHDV emergence in Australia (Strive et al., 2009). A new non-pathogenic lagovirus, named rabbit calicivirus Australia 1 (RCV-A1), was identified by molecular methods by Strive et al. (2009), in

the gut of apparently healthy young wild rabbits. The virus is responsible for a predominantly enteric infection (Jahnke et al., 2010). The RCV-A1 strains' non-lethality was experimentally confirmed (Strive et al., 2010).

Complete viral genome sequencing and phylogenetic analysis revealed that RCV-A1 formed a separate lineage among the rabbit caliciviruses, constituting a new member of the genus *Lagovirus* (Strive et al., 2009)(Jahnke et al., 2010). RCV-A1 viruses are more distantly related to RHDV than the European non-pathogenic lagovirus and were shown to confer only partial protection against RHDV (Jahnke et al. 2010).

RCV-A1 emergence coincided with the introduction of the wild rabbits in Australia in 1850 (Jahnke et al., 2010). RCV-A1 strains diversified then into several geographically distinct lineages, forming a monophyletic group subdivided into six clades, clustering mostly according to their place of sampling (Jahnke et al., 2010). RCV-A1 was effectively disseminated via the initial rabbits' dispersal, but once a rabbit population was established, virus migration rate decreased (Jahnke et al., 2010).

2.2.5. Michigan rabbit calicivirus (MRCV), US

Bergin et al. (2009) reported a novel rabbit calicivirus in Michigan State. The case dated back to 2001 and occurred in New Zealand White rabbits (*O. cuniculi*) in a private rabbitry. Infected rabbits showed clinical signs and pathologic findings suggestive of RHD and the virus was designated MRCV. MRCV was shown to be more closely related to the non-pathogenic rabbit calicivirus than to pathogenic strains. This virus was classified as a novel lagovirus distinct from RHDV, RCV and EBHSV, and the first lagovirus other than RHDV detected in US rabbits. (Abrantes and Esteves, 2010) carried out a phylogenetic analysis including the Ashigton strain and RCV-A1 viruses, not included in the analysis performed by Bergin et al. (2010). The authors concluded that MRCV was a new variant of the NP-LVs.

Considering the percentage of affected animals in this outbreak and that the authors were not able to reproduce clinical disease in specific pathogen-free (SPF) rabbits, MRCV was considered to be of low pathogenicity, and the onset of clinical disease would depend on health status, age, or individual susceptibility of the host. In marked contrast to other low pathogenicity caliciviruses such as RCV, that only replicate in the intestine, MRCV was detected in the liver. Whether MRCV could induce some protection against RHDV infection was not investigated. The low MRCV pathogenicity and the presence of viral RNA in the liver constituted new features among the nonpathogenic RCV-like group (Abrantes and Esteves, 2010).

2.2.6. The 06-11 strain from France

Le Gall-Reculé et al. (2011b) identified a new infectious rabbit lagovirus which differed from RHDV in tissue tropism (small intestine only), pathogenicity and on the VP60 capsid protein. This new virus was detected in healthy domestic rabbits, older than 2 months, and designated as the 06-11 strain.

The 06-11 virus was shown to be highly infectious but non-lethal for rabbits, as experimentally demonstrated in specific pathogen free (SPF) rabbits. However, in the absence of histological data the entire lack of pathogenicity of this virus was not demonstrated.

Regarding RHDV cross-protection, 06-11 induced antibodies that did not to protect against RHDV irrespective of their titre. In fact, previous studies confirmed the presence of non-protective antibodies in wild rabbits that died from RHDV, probably induced by NP-LV infection (Marchandeau et al., 2005)(Le Gall-Reculé et al., 2011b).

Phylogenetic analysis including the 06-11 virus and other NP-LV as well as RHDV, showed that the 06-2011 strain grouped with RCV and the Ashignton strain. Notwithstanding, the three viruses showed significant genetic variation from each other and from the RHDV sequences. When the *vp60* gene sequences were compared, 06-11 exhibited a closer relation to RCV than to RHDV or RCV-A1 showing however the highest nucleotide and amino acid identity with the Ashington strain. The phylogenetic tree topology obtained by Le Gall-Reculé et al. (2011) suggested that RCV and the 06-11/Ashington lineage emerged long before pathogenic RHDV lineage divergence, with RCV emerging prior to the 06-11/Ashington lineage. The mean time to the most recent common ancestor (TMRCA) of RHDV and NP-LV strains was estimated to be over 80 (Kinnear and Linde, 2010) or 200 years ago (Kerr et al., 2009)(Le Gall-Reculé et al., 2011b), in accordance with Moss et al., (2002) and Kerr et al., (2009). Nonetheless, the significant genetic variation found between 06-11 and the Ashington strain suggests a putative independent evolution for both viruses (Le Gall-Reculé et al., 2011).

Taken together, the date obtained by Le Gall-Reculé et al. (2011) indicated a considerable divergence between these different NP-LV strains.

Study 8

What are the phylogenetic relations between the RHDV2 strains circulating in Azores islands and in Europe?

Rabbit haemorrhagic disease virus 2 (RHDV2) outbreak in Azores: disclosure of common genetic markers and phylogenetic segregation within the European strains.

Margarida D. Duarte[1], Carina L. Carvalho[2], Susana Bernardo[3], Sílvia V. Barros[3], Sandra Benevides[3], Lídia Flor[3], Madalena Monteiro[1], Isabel Marques[4], Ana M. Henriques[1], Sílvia S. Barros[1], Teresa Fagulha[1], Fernanda Ramos[1], Tiago Luís[1], Miguel Fevereiro[1]

[1]Instituto Nacional de Investigação Agrária e Veterinária (INIAV), Lisboa, Portugal; [2]Instituto de Ciências Agrárias e Ambientais Mediterrânicas (ICAAM), Universidade de Évora, Évora, Portugal; [3]Laboratório Regional de Veterinária, Açores, Portugal; [4]Instituto Gulbenkian de Ciência (IGC), Oeiras, Portugal;

Infection Genetics and Evolution 35:163-71 doi: 10.1016/j.meegid.201

Infection, Genetics and Evolution 35 (2015) 163–171

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

Rabbit haemorrhagic disease virus 2 (RHDV2) outbreak in Azores: Disclosure of common genetic markers and phylogenetic segregation within the European strains

Margarida Duarte [a,*], Carina Carvalho [b], Susana Bernardo [c], Sílvia Vanessa Barros [c], Sandra Benevides [c], Lídia Flor [c], Madalena Monteiro [d], Isabel Marques [e], Margarida Henriques [a], Sílvia C. Barros [a], Teresa Fagulha [a], Fernanda Ramos [a], Tiago Luís [a], Miguel Fevereiro [a]

[a] Instituto Nacional de Investigação Agrária e Veterinária (INIAV), Virology Laboratory, Rua General Morais Sarmento, 1500-311 Lisbon, Portugal
[b] Instituto de Ciências Agrárias e Ambientais Mediterrânicas – ICAAM, Universidade de Évora, Núcleo da Mitra, 7000 Évora, Portugal
[c] Laboratório Regional de Veterinária dos Açores, Vinha Brava 9700-236, Angra do Heroísmo, Azores, Portugal
[d] Instituto Nacional de Investigação Agrária e Veterinária (INIAV), Pathology Laboratory, Rua General Morais Sarmento, 1500-311 Lisbon, Portugal
[e] Instituto Gulbenkian de Ciência, Rua da Quinta Grande, 6, 2781-156 Oeiras, Portugal

ARTICLE INFO

Article history:
Received 13 April 2015
Received in revised form 28 July 2015
Accepted 3 August 2015
Available online 4 August 2015

Keywords:
Rabbit haemorrhagic disease virus
RHDV2
RHDVb
Azores
Portugal
Single nucleotide polymorphisms
SNPs
Phylogenetic analysis
Histopathology

ABSTRACT

Rabbit haemorrhagic disease virus 2 (RHDV2) is widespread in several countries of Western Europe, but it has not been introduced to other continents. However, between late 2014 and early 2015, the presence of RHDV2 was confirmed outside of the European continent, in the Azores, initially in the islands of Graciosa, Flores, S. Jorge and Terceira. In this study we report the subsequent detection of RHDV2 in wild rabbits from the islands of Faial, St. Maria and S. Miguel, and display the necropsy and microscopic examination data obtained, which showed lesions similar to those induced by classical strains of RHDV, with severe affection of lungs and liver. We also disclose the result of a genetic investigation carried out with RHDV2 positive samples from wild rabbits found dead in the seven islands. Partial *vp60* sequences were amplified from 27 tissue samples. Nucleotide analysis showed that the Azorean strains are closely related to each other, sharing a high genetic identity (>99.15%). None of the obtained sequences were identical to any RHDV2 sequence publically known, hampering a clue for the source of the outbreaks. However, Bayesian and maximum likelihood phylogenetic analyses disclosed that Azorean strains are more closely related to a few strains from Southern Portugal than with any others presently known. In the analysed region comprising the terminal 942 nucleotides of the *vp60* gene, four new single nucleotide polymorphisms (SNP) were identified. Based on the present data, these four SNPs, which are unique in the strains from Azores, may constitute putative molecular geographic markers for Azorean RHDV2 strains, if they persist in the future. One of these variations is a non-synonymous substitution that involves the replacement of one amino acid in a hypervariable region of the capsid protein.

© 2015 Elsevier B.V. All rights reserved.

1. Introduction

Rabbit haemorrhagic disease (RHD) is a highly contagious and often lethal systemic infection in wild and domestic rabbits caused by the pathogenic rabbit haemorrhagic disease viruses RHDV and RHDV2, which were identified for the first time in 1984 (Lui et al., 1984) and 2010 (Le Gall-Recule et al., 2011a), respectively. These naked and single stranded RNA viruses are closely related, sharing 82.4% of genetic identity (Le Gall-Recule et al., 2013).

RHDV and RHDV2 belong to the Lagovirus genus of the Caliciviridae family (Le Gall-Recule et al., 2011a) along with the European brown hare syndrome virus (EBHSV), and non- or moderately-pathogenic lagoviruses (NP-LV, in accordance with the nomenclature suggested by (Le Gall-Recule et al., 2011b). The NP-LV group includes the RCV-A1 strains from Australia that are more distantly related to RHDV (Jahnke et al., 2010), the RCV strain from Italy, which was the first non-pathogenic strain being described (Capucci et al., 1996) and other lagoviruses, often designated RCV-like due to their closer phylogenetic proximity with the RCV strain. These encompass the Lambay strain from Ireland (Forrester et al., 2007), the MRCV strain from the USA (Bergin

* Corresponding author.
E-mail address: margarida.duarte@iniav.pt (M. Duarte).

http://dx.doi.org/10.1016/j.meegid.2015.08.005
1567-1348/© 2015 Elsevier B.V. All rights reserved.

et al., 2009), and the strain 06–11 from France (Le Gall-Recule et al., 2011b). The pathogenicity of the Ashington strain from UK (Moss et al., 2002), also a RCV-like virus, was never demonstrated experimentally (Le Gall-Recule et al., 2011b).

The clinical characteristics induced by RHDV and RHDV2 infections may differ in terms of disease duration, mortality rates and in the occurrence of sub acute or chronic forms, which are more frequent in RHDV2 infected rabbits (Le Gall-Recule et al., 2013). Regarding the pathological aspects of the disease, the lesions induced by RHDV have been detailed by several authors (Lavazza et al., 1996; McIntosh et al., 2007) but the characterisation on the lesions developed after RHDV2 infection is still limited. *Post-mortem* examination of RHDV2 infected rabbits revealed macroscopic lesions consistent with haemorrhages in several organs including heart, trachea, thymus, lungs, liver, kidneys, and gut, as well as jaundice (Dalton et al., 2012). The liver appearance was described as pale and congested (Lopes et al., 2015). Histopathologic descriptions refer haemorrhagic pneumonia and tracheitis, congestion of the liver and diffuse necrotizing hepatitis. Areas of focal necrosis were also described in the intestinal *villi* in the small intestine (Dalton et al., 2012).

Based on their phylogenetic relationships, RHDV strains were classified in distinct genetic groups (designated G1 to G6, the latter also referred as RHDVa) (Le Gall-Recule et al., 2003). The alignment of the VP60 sequences from RHDV strains, representing these six genogroups, revealed seven domains (V1 to V7) (Wang et al., 2013) in which the highest amino acid variability is observed. In NP-LVs the variability exceeds these seven domains, encompassing the complete capsid protein (Le Gall-Recule et al., 2013).

Molecular studies also revealed that most of the strains that circulated in Portugal mainland prior to 2012 belonged, or were more related to, genogroup G1 (Abrantes et al., 2012; Duarte et al., 2014b; Muller et al., 2009), although strains from genogroup G6 were also identified (Muller et al., 2009; Duarte et al., 2014b). However, Bayesian phylogenetic analysis clustered together six strains from the Azores, obtained between 2006 and 2013, into genogroup G5 (Duarte et al., 2014b), which was never reported in the mainland.

RHDV2 recently emerged in France 2010 in European rabbits and the lack of immunological cross protection induced by the previous contact with classical strains facilitated the spread of the disease among domestic and wild rabbits (Le Gall-Recule et al., 2013, 2011a). In the following years the disease spread to Italy (Le Gall-Recule et al., 2013; Puggioni et al., 2013), Spain (Dalton et al., 2012), Portugal (Abrantes et al., 2013), England and Wales (Westcott et al., 2014), Scotland (Baily et al., 2014), and Germany (information on the FLI, 10|21|2013).

Outside the European continent, RHDV2 was first detected between late 2014 and early 2015 in the Azores (Duarte et al., 2015a). This archipelago, located in the middle of the North Atlantic Ocean comprises nine islands, namely Flores and Corvo (Western group), Faial, S. Jorge, Pico, Terceira and Graciosa (Central group) and St. Maria and S. Miguel (Eastern group).

Here we report the presence of RHDV2 in the islands of Faial, St. Maria and S. Miguel for the first time and the result of an investigation involving the partial molecular characterisation of the *vp60* gene of RHDV2 representative strains from seven islands (Flores ($n = 3$), Graciosa ($n = 6$), Terceira ($n = 5$), S. Jorge ($n = 4$), Faial ($n = 4$), St. Maria ($n = 1$) and S. Miguel ($n = 4$). The molecular study aimed to determine the level of genetic relationship between the RHDV2 strains from the seven outbreaks in Azores and to investigate their possible source of infection. In this study, we also unveil the results of the necropsy of 32 RHDV2-positive wild rabbits and the histopathological data from 10 specimens.

2. Materials and methods

2.1. Samples

Information on the geographic origin and the date of collection of the samples used in this study is described in Table 1. Liver and lung samples were collected during necropsy.

2.2. Necropsy and histopathological examination

Necropsy and anatomopathological examinations were performed at the Regional Veterinary Laboratory of Azores.

Liver and lung samples were fixed in 10% buffered formalin and embedded in paraffin by standard procedures. Five micrometre-thick sections were stained with haematoxylin and eosin (H&E) and examined using light microscopy (Cook, 1997).

2.3. Virological examination

For virological examination, liver samples collected from all rabbits and lung samples from nine animals were homogenised with phosphate buffer saline (PBS) and clarified at 3000*g* for 5 min.

DNA and RNA were extracted from 200 μl of clarified supernatant, corresponding to 50–60 mg of tissue, in a BioSprint 96 nucleic acid extractor (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Samples were tested for RHDV2 RNA using the RT-qPCR previously described by (Duarte et al., 2015b). Screening for RHDV was performed by sequencing analysis of the amplicons obtained with primers RC-9 and RC-10, published by (Tham et al., 1999). The presence of myxoma virus DNA was ruled out with the method published by (Duarte et al., 2014a).

2.4. Sequencing analysis and multiple alignments

Amplification of the 3′ end of the *vp60* gene of RHDV2 strains was accomplished using two forward primers (717-F and RC-9F) and one reverse primer (RC-10R) described in Table 2. The reactions were performed with 10 μl of RNA and 25 pmol of each primer, using the OneStep RT-PCR kit (Qiagen), according to the manufacturer's protocol. Amplification conditions included a reverse transcription step at 50 °C for 30 min, an initial denaturation at 95 °C for 5 min, followed by 50 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 30 s and extension at 72 °C for 30 s.

The fragments were excised from agarose gels after electrophoresis and purified with the NZYGelpure (Nzytech genes and enzymes, Lisbon, Portugal).

Sequencing was performed with the amplifying primers as well as with primers 1190-F and 1404-R (Table 2), using a BigDye™ Terminator cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Partial *vp60* nucleotide sequences of 27 RHDV2 strains were determined on an automated 3130 Genetic Analyzer system (Applied Biosystems, Foster City, CA, USA) (Table 1). Thirteen sequences were submitted to GenBank (Table 1). Multiple alignments were generated by CLUSTALW (Thompson et al., 1994) and edited in GeneDoc (version 2.7, Nicholas et al., 1997) for the calculation of the percentage similarity.

2.5. Phylogenetic analysis

Except for the Azorean strains, all the *vp60* sequences analysed were retrieved from GenBank. Multiple sequence alignments were generated by CLUSTAL W (Thompson et al., 1994) and two sets of

Table 1
Information on the geographic origin and date of collection of the samples used for this study and accession numbers of the vp60 sequences submitted to GenBank.

Strain	Island of origin	Locality	Collection date	Accession numbers	Region sequenced (vp60 gene)	SNPs*
13647PT15	Faial	Praia Almoxarife	15–20.01.2015	KP862922	753-1740	1, 2, 3, 4
13648PT15	Faial	Praia Almoxarife	15–20.01.2015	KP862921	798-1740	1, 2, 3, 4
13649PT15	Faial	Flamengos	15–20.01.2015	KP862923	798-1740	1, 2, 3, 4
13650PT15	Faial	Norte	15–20.01.2015	KP862924	798-1740	1, 2, 3, 4
6628PT15	Terceira	Serra do Cume	11.01.2015	KP862925	771-1740	1, 2, 3, 4
6629PT15	Terceira	Serra do Cume	11.01.2015	KP862926	763-1740	1, 2, 3, 4
5145PT15	Terceira	Serra do Cume	08.01.2015	KP862927	1444-1740	3, 4
2844PT15	S. Jorge	S. Amaro	05.01.2015	*Not submitted*	750-1740	1, 2, 3, 4
2845PT15	S. Jorge	S. Amaro	05.01.2015	*Not submitted*	750-1740	1, 2, 3, 4
2846PT15	S. Jorge	Rosais	05.01.2015	*Not submitted*	750-1740	1, 2, 3, 4
2847PT15	S. Jorge	Norte Grande	05.01.2015	KP862928	1380-1740	3, 4
2133PT15	Flores	St. Cruz Flores	06.01.2015	*Not submitted*	1444-1740	3, 4
2134PT15	Flores	Fazenda, Lajes	02.01.2015	*Not submitted*	1444-1740	3, 4
2135PT15	Flores	Fazenda, Lajes	02.01.2015	*Not submitted*	1444-1740	3, 4
630PT15	Terceira	Golfo	02.01.2015	*Not submitted*	750-1740	1, 2, 3, 4
631PT15	Terceira	Golfo	02.01.2015	*Not submitted*	750-1740	1, 2, 3, 4
227251PT14	Graciosa	Serra Branca	18.12.2014	KP862929	76-1740	1, 2, 3, 4
227252PT14	Graciosa	Serra Branca	18.12.2014	KP862930	108-1740	1, 2, 3, 4
227253PT14	Graciosa	Serra Branca	18.12.2014	KP862931	784-1740	1, 2, 3, 4
227254PT14	Graciosa	Serra Branca	18.12.2014	KP862932	746-1740	1, 2, 3, 4
227255PT14	Graciosa	Serra Branca	18.12.2014	KP862933	745-1740	1, 2, 3, 4
227256PT14	Graciosa	Serra Branca	18.12.2014	*Not submitted*	750-1740	1, 2, 3, 4
28695PT15	St. Maria	Espirito Santo	09.01.2015	*Not submitted*	1444-1740	3,4
29149PT15	S. Miguel	Ponta da Graça	08.02.2015	*Not submitted*	1444-1740	3,4
29150PT15	S. Miguel	Nordeste	08.02.2015	*Not submitted*	1444-1740	3,4
29151PT15	S. Miguel	Nordeste	08.02.2015	*Not submitted*	1444-1740	3,4
29152PT15	S. Miguel	Nordeste	08.02.2015	*Not submitted*	1444-1740	3,4

* Azorean specific markers observed in the nucleotide sequences obtained.

Table 2
Information regarding the primers used in this study.

Primer	Sequence (5′–3′)	Location* in the vp60 gene (KJ683896)	Use	Size of the amplicon (bp)	Region of the amplicon sequenced	Reference
717-F	CGCAGATCTCCTCACAACCC	717	Amplif/Seq	1048	798 to 1740 (942nt)	*This study*
RC-9F	ATCATGTTCGCGTCTGTCGTCAGG	1381	Amplif/Seq	384	1407 to 1740 (333nt)	Tham et al. (1999)
RC-10R	GCGCCTGCAAGTCCCAATCC	27nt downstream the stop codon	Amplif/Seq			Tham et al. (1999)
1190-F	CTCAGATTGTTGCCAAGTCC	1190	Seq	–		*This study*
1404-R	CCTGACGACAGACGCGAA	1404	Seq	–		*This study*

* Location refers to the 5′ end of the primers; Amplif (Amplification); Seq (Sequencing).

alignments were considered for the phylogenetic analyses. The first was composed by 122 942-nucleotide long sequences, representing 54.1% of the *vp60* complete gene. In the second alignment, six additional *vp60* sequences of strains from Flores, St. Maria and S. Miguel islands were added. The region included in this last alignment was restricted to the terminal 333 VP60 encoding nucleotides. The multiple sequence alignments were manually corrected with Jalview, Version 2.0.1 (Waterhouse et al., 2009) removing internal gaps and unmatched ends, to maximise genetic similarities. Phylogenetic trees were inferred by Maximum Likelihood (ML) and Bayesian methods, PhyML 3.0 (Guindon et al., 2010) and MrBayes v.3.2.1 (Huelsenbeck et al., 2001; Ronquist and Huelsenbeck, 2003) programs, respectively. For the ML criterion the substitution model HKY85 was selected (Hasegawa et al., 1985) assuming an estimated proportion of invariant sites and 4 gamma-distributed rate categories to account for rate heterogeneity across sites. Initial trees were constructed using BIONJ, and nearest neighbour interchange (NNI) with 1000 bootstrap replicates. CONSENSE program from the PHYLIP package (Felsenstein, 2004) was used for the consensus tree. For the Bayesian analysis a Markov chain Monte Carlo (mcmc) simulation technique was carried out to approximate the posterior probabilities of trees (Huelsenbeck et al., 2001; Ronquist and Huelsenbeck, 2003). The evolutionary GTR model (Tavaré, 1986) (nst = 6) was selected with gamma-distributed rate variation across sites and a proportion of invariable sites (rates = invgamma). The analysis was initiated using a random tree from the dataset with four chains running simultaneously for 10×10^6 generations, sampling every 100 generations. The first 25% trees were discarded (burn-in) and a majority rule consensus tree was generated from the remaining trees.

The graphical representation and edition of the phylogenetic trees were performed with FigTree v1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/). Only support values equal or greater than 70% of bootstrap (BS) and 0.70 of posterior probability (PP) are shown in the trees. Sequence Z69620, from a European brown hare syndrome virus, was chosen as outgroup to root the trees.

3. Results

3.1. Gross and histopathologic examination of wild rabbits positive to RHDV2 revealed lesions identical to those induced by classical strains of RHDV

Thirty-two wild rabbits were necropsied. No external parasites were found. Nostrils with severe congestion and hemorrhages were observed in 25 of the animals (83.3%) and hematochezia in three (10.0%). Lungs were the most commonly affected organ showing moderate to severe congestion, petechial or larger

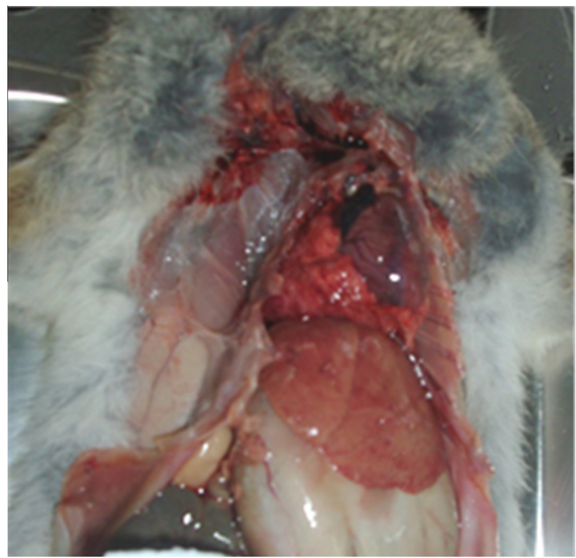


Fig. 1. Macroscopic examination of a wild rabbit from Faial positive to RHDV2 showing pale liver and congested lungs.

haemorrhages irregularly distributed and affecting partial or complete lobes (Fig. 1). In all necropsied animals the tracheal mucosa, thymus, kidneys and spleen also showed congestion. Livers were slightly enlarged, soft and pale (Fig. 1).

Most of the specimens were received frozen and/or under significant decomposition, limiting the anatomopathological examination to ten animals. In those, histopathology of the liver revealed hydropic degeneration ($n = 10$, 100%). Most of the animals ($n = 8$, 80%), exhibited acute and widespread necrosis with dissociation of the hepatic cords (Fig. 2). Hepatocytes showed hypereosinophilic cytoplasm and pyknosis, karyorrhexis and karyolysis (Fig. 2A and B). In two rabbits, hydropic degeneration was the only lesion observed in the liver. Acidophilus intracytoplasmic bodies were present in degenerated hepatocytes of two rabbits (20%) (Fig. 2B). Histopathology of the lungs revealed diffused congestion (Fig. 3A), alveolar oedema and moderate to severe intralveolar haemorrhage in all animals (100%). Disseminated intravascular coagulation (DIC) was present in the capillaries and small arterioles of the lung in five rabbits (50%) or in both the lungs (Fig. 3A) and liver (Fig. 3B) in one animal (10%).

3.2. Molecular characterisation and comparison of partial vp60 sequences from Azorean strains shows high level of nucleotide similarity among strains regardless the island of origin

All the samples from wild rabbits originated in the seven Azorean islands tested positive to RHDV2 (32/32, 100%) with the standardised RT-qPCR used in this investigation (Duarte et al., 2015b). The Cq values obtained for the liver samples ranged between 9.20 and 26.15, which corresponds approximately to 9×10^4 to 9×10^9 viral particles in the PCR test.

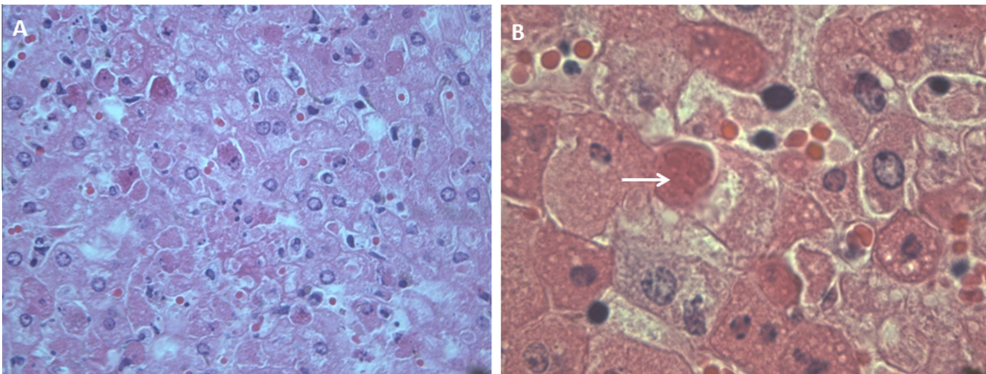


Fig. 2. Histopathological lesions in the liver of a wild rabbit from Graciosa positive to RHDV2. (A) Necrotic hepathocytes with karyorrhexis and karyolysis (H&E, 400×). (B) Degenerated hepatocytes with acidophilus intracytoplasmic bodies (arrow) (H&E, 1000×).

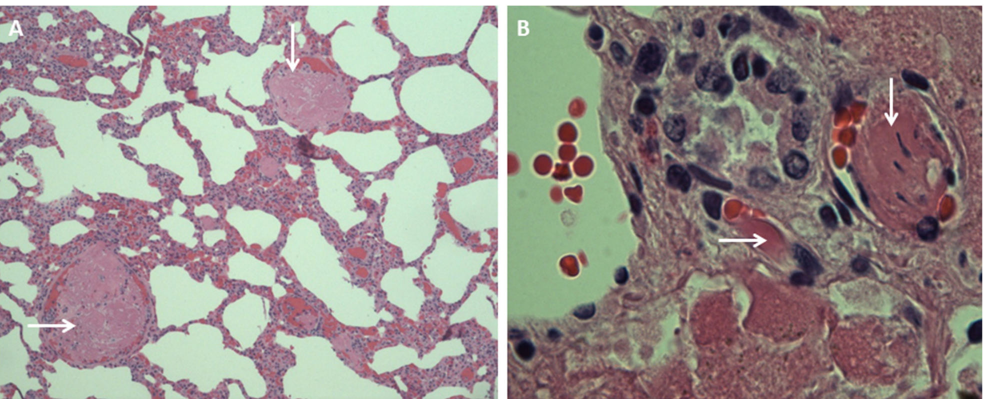


Fig. 3. Histopathology of the lung and liver of a wild rabbit from St. Maria positive to RHDV2 showing thrombus in the capillaries and small arterioles (arrows). (A) Lung (H&E, 100×), (B) Liver (H&E, 1000×).

Table 3
Single nucleotide polymorphisms (SNPs) found in the vp60 gene (942-1740 nt) of RHDV2 strains.

		SNPS UNIQUE IN THE AZOREAN STRAINS				SNPS PREDOMINANT IN THE AZOREAN STRAINS				
		SNP1	SNP2	SNP3	SNP4	SNP5	SNP6	SNP7	SNP8	SNP9
	Nucleotide position in *vp60* gene	930	1294	1689	1704	919	1045	1464	1491	1635
	Polymorphism	A>G	A>G	C>T	C>T	A>G	G>A	A>G	A>G	T>C
Country	Position of the SNP within the codon	3th	1st	3th	3th	1st	1st	3th	3th	3th
(Collection date)	Position of the amino acid encoded	310	432	563	568	307	349	488	497	545
	Type of substitution	syn	non-syn	syn	syn	non-syn	non-syn	syn	syn	syn
France (2010)	SNP/codon	TCA	AGT	AGC	AAC	AGC	GTC	GGA	GGA	ATT
	Amino acid encoded	Ser	Ser	Ser	Asn	Ser	Val	Gly	Gly	Ile
	Number of sequences analysed	6	6	6	6	6	6	6	6	6
	Prevalence of polymorphism (%)	100	100	100	100	100	100	100	100	100
Italy (2011)	SNP/codon	TCA	AGT	AGC	AAC	AGC	GTC/ATC	GGA	GGA/GGG	ATT
	Amino acid encoded	Ser	Ser	Ser	Asn	Ser	Val/Ile	Gly	Gly	Ile
	Number of sequences analysed	8	8	8	8	8	6/2[a]	8	6/2[b]	8
	Prevalence of polymorphism (%)	100	100	100	100	100	75/25	100	75/25	100
Spain (2011–2012)	SNP/codon	TCA	AGT	AGC	AAC	AGC/GGC	GTC/ATC	GGA/GGG	GGA	ATT/ATC
	Amino acid encoded	Ser	Ser	Ser	Asn	Ser	Ile	Ser	Gly	Ile
	Number of sequences analysed	7	7	7	7	6/1[c]	6/1[d]	6/1[e]	7	6/1[d]
	Prevalence of polymorphism (%)	100	100	100	100	85.7/14.3	85.7/14.3	85.7/14.3	100	85.7/14.3
Mainland, Portugal (2013–2014)	SNP/codon	TCA	AGT	AGC	AAC	AGC	GTC/ATC	GGA/GGG	GGA/GGG	ATT
	Amino acid encoded	Ser	Ser	Ser	Asn	Ser	Val/Ile	Gly	Gly	Ile
	Number of sequences analysed	56	56	56	56	56	56	47/9[f]	55/1[g]	56
	Prevalence of polymorphism (%)	100	100	100	100	100	100	83.9/16.1	98.2/1.8	100
Azores, Portugal (2014–2015)	SNP/codon	TCG	GGT	AGT	AAT	GGC	ATC	GGG	GGG	ATC
	Amino acid encoded	Ser	Gly	Ser	Asn	Gly	Ile	Gly	Gly	Ile
	Number of sequences analysed	20	12	27	27	20	20	27	27	27
	Prevalence of polymorphism (%)	100	100	100	100	100	100	100	100	100

a – KC907712, JX106022; b – KC907712, JX106022; c – KP090976; d – KP129398; e – KP090976; f – KM115667-75; g – KM115693.

All the samples tested negative to myxoma virus (0/32, 0%) by the method described by (Duarte et al., 2014a). None of the animals were positive to RHDV.

The BLAST analysis of the partial *vp60* sequences obtained from 27 samples from wild rabbits originated in the seven islands, showed high similarity with RHDV2 strains from the European continent. A genetic identity of 99.36% to 100% was inferred by the nucleotide variability found in the alignment of the 3′end-942 bases of 11 Azorean sequences. In the alignment of a shorter region, limited to the terminal 333 bases of 17 *vp60* sequences, an identity of 99.4% to 100% was observed.

3.3. New single nucleotide polymorphisms (SNPs) in the vp60 gene at positions 930, 1294, 1689 and 1704 found in the strains from Azores (2014-February 2015)

The nucleotide alignment of the partial *vp60* sequences of Azorean strains with all the RHDV2 sequences presently known revealed four new SNPs in the insular strains at nucleotide positions 930, 1294, 1689 and 1704. Other SNPs, at positions 919, 1045, 1464, 1491 and 1635, were found to be predominant in the strains from the Azores although also present in strains from Spain, Portugal mainland or Italy (Table 3). Apart from the Azorean strains, the A/G SNP at position 919 is only found in a strain from Spain (KP090976). Moreover, the combined presence of these 9 SNPs was only detected in the Azorean strains.

3.4. Comparison of Azorean strains with strains originated worldwide, revealed no obvious similarity with any European strain

Despite the high nucleotide identities observed between the Azorean strains and strains from Portugal mainland (96.7–98.3%) and Spain (95.75–98.2%), no absolute similarity was found between any strain from Azores and a RHDV2 sequence from other geographic origin.

3.5. Phylogenetic analysis discloses segregation from European RHDV2 strains

The Bayesian and maximum likelihood trees generated with the *vp60* terminal 942 nucleotides of 11 Azorean strains, RHDV2 strains from Europe, representatives from the classical genogroups (G1–G6) and the non-pathogenic strains RCV (X96868), NP-LV 06–11 (AM268419) and RCV-A1 (GU368890, GU368894, GU368919), showed very similar topologies. A few exceptions included sequence HE800529 that in the ML analysis grouped with other strains from France, while in the Bayesian analysis has a more close relation with the Iberian strains.

RHDV2 strains separated clearly from classical RHDV and the non-pathogenic lagoviruses RCV and NP-LV 06–11 (BS = 82%, node A, Fig. 4A), forming a vast monophyletic group strongly supported by the two phylogenetic analyses (BS = 100%, PP = 1, node B). Within the RHDV2 cluster, most of the strains from France and Italy collected until 2012 show to be more closely related with each other than with the strains with other European geographic origins. All Iberian strains appear to have derived from a common ancestor shared also by a unique non-Iberian strain (KC741409) collected from a *Lepus corsicanus* specimen in mainland Italy (BS = 71%, PP = 1, node C, Fig. 4A). Along with two strains from Spain (KP129395, KP129396), all strains from Portugal appear to share a common ancestor (BS = 70%, node D). The Azorean strains formed a strongly supported monophyletic group (BS = 100%, PP = 1, node F), and segregated from the remaining strains originated in continental Portugal. Also, the Bayesian and ML analysis revealed that the Azorean strains appear to be more closely related with a subgroup of strains from Mértola (subregion Baixo Alentejo) and the Algarve. Those five strains originated in the South of the country grouped together consistently in the two trees with strong bootstrap and posterior probability values of 100% and 1, respectively (Fig. 4A, node G). However, the genetic proximity between the Azorean strains and these five Southern RHDV2 strains was supported only weakly by a bootstrap value of 48.1% and a low posterior probability of 0.67 (node E, *values not shown in* Fig. 4).

The phylogenetic analyses based on a smaller region (333 nucleotide-long) allowed us to extend the analysis to a total of 17 sequences from Azores. The overall topology of trees reproduced the relative position of the subgroups defined in the former. A close up of the Azorean cluster, obtained with the ML shorter alignment (333 nucleotides), is presented in Fig. 4B. Regarding the relative phylogenetic relations between the Azorean isolates, the ML analysis further resolved the distribution of the sequences within this branch (Fig. 4B), overcoming the Bayesian approach (result not shown). However, only a few nodes were supported by BS values higher than 70% (Fig. 4B) and no obvious relation could be established between the island of origin and the distribution of these sequences in the Azorean cluster. Also in this 333 nt based analysis, high bootstrap and posterior probability values supported the clustering of the Azorean sequences (BS = 94.4, PP = 1, node F) as well as of the sequences from Southern Portugal (BS = 94, PP = 1, node G). The relative topology of these branches agreed with the branching obtained in the 942 nt based analysis.

The consistency of the inferences performed with the longer (942 nt) and shorter (333 nt) alignments revealed that the nucleotide variability within the 333-nucleotide long region harbours enough discriminatory power to allow phylogenetic analysis. In this alignment six additional sequences from Flores, St. Maria and S. Miguel were included. The genetic distances calculated by the Kimura 2-parameters (K80) and F84 models ranged from 0.00000 and 0.006407 and 0.006409, respectively (*results not shown*). However, the highest distance was found between sequence KP862930 and KP862924 from the Graciosa and Faial islands, respectively.

4. Discussion and conclusions

The anatomopathological examination of specimens victimised by RHDV2 in the Azorean islands allowed us to confirm the infection induced lesions previously described by other authors (Dalton et al., 2012; Lopes et al., 2015) and to further extend their detailed description. In fact, our analysis showed that the macroscopic lesions observed in RHDV2-positive wild rabbits from Azores match typical RHDV lesions. For instance, they were similar to those described in rabbits infected with RHDV classical strains (McIntosh et al., 2007), reporting pale liver and multiple infarcts and haemorrhages throughout the lungs. Moreover, our observations were in accordance with the histopathological exam of classical RHDV victimised rabbits, where severe hepatic necrosis characterised by disassociation of the hepatic cords, cellular swelling, hyper eosinophilia and hepatocellular vacuolar changes (pyknosis, karyorrhexis and karyolysis) were described (McIntosh et al., 2007). Degenerative hepatocytes containing intracytoplasmic acidophilic bodies were also observed in both infections (Fig. 3). As for the fatally RHDV infected rabbits (McIntosh et al., 2007), lungs from RHDV2 positive rabbits from Azores, showed pulmonary congestion and haemorrhage.

Despite the distinct clinical characteristics of the disease induced by RHDV2 and RHDV regarding the age of the affected rabbits, disease duration, mortality rates and the occurrence of subacute/chronic forms (Le Gall-Recule et al., 2013), the similarities between the lesions induced by both viruses, hamper the

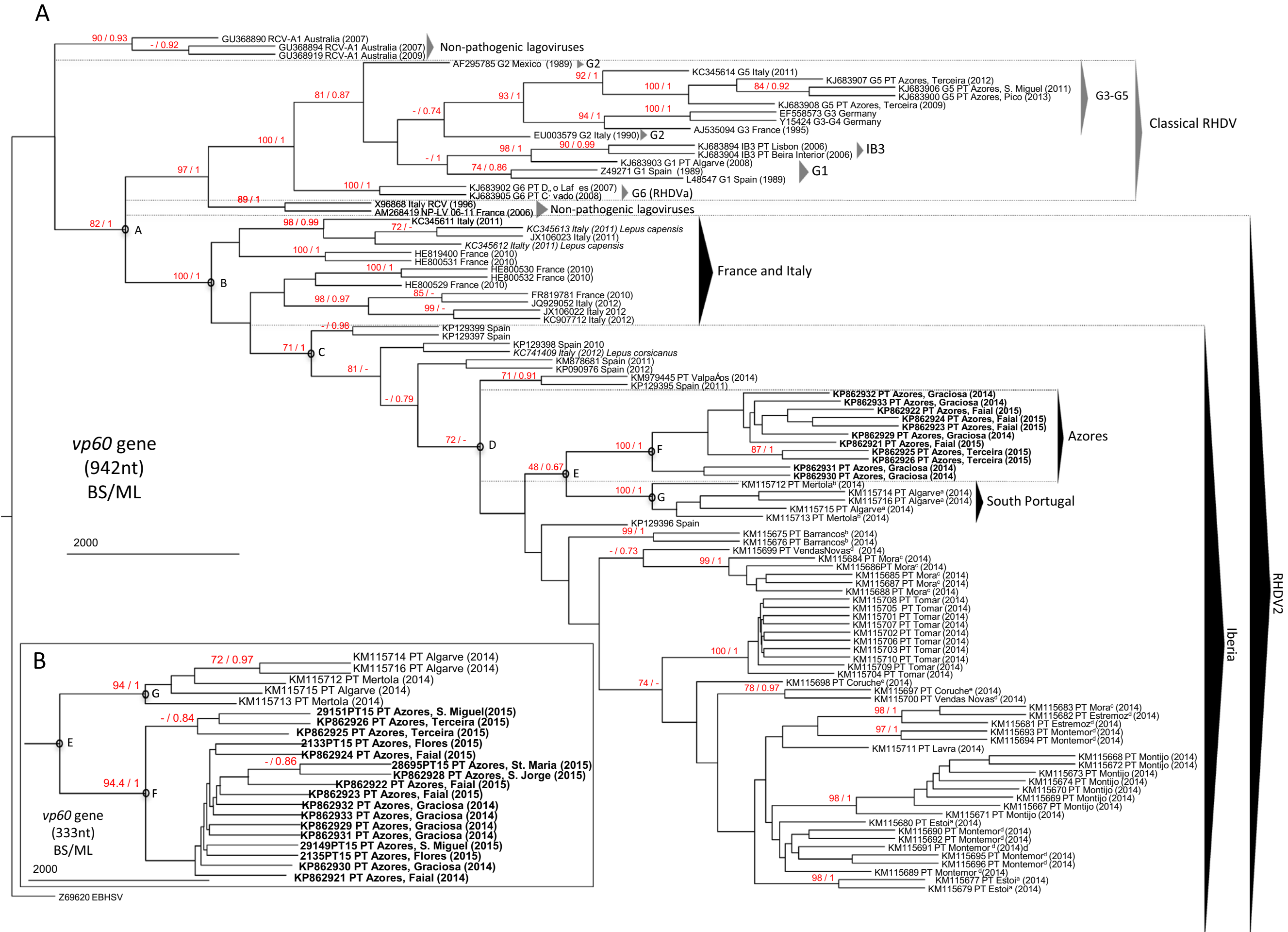


Fig. 4. (A) Phylogenetic tree derived from maximum molecular likelihood based on partial *vp60* sequences (3′ terminal 942 nucleotides). (B) Close up of the Azorean branch obtained in the phylogenetic tree derived from maximum molecular likelihood based on partial *vp60* sequences (5′ terminal 333 nucleotides). Bootstrap (BS) values from maximum likelihood analysis followed by posterior probability (PP) values from Bayesian analysis are added to the left of a node (BS/PP). Only support values equal or greater than 70% of bootstrap (BS) and 0.70 of posterior probability (PP) are shown in the tree. Sequence Z69620 (EBHSV) was chosen as outgroup to root the tree. The NUTS3 (Territorial Statistics based on Administrative and Autonomous Regions) classification of the Southern strains are indicated by superscript letters after the geographic origin of the sequences (a-Algarve; b-Baixo Alentejo; c-Alto Alentejo; d-Alentejo Central; e-Lezíria do Tejo).

differential diagnosis through anatomopathological examination. Therefore, the differentiation between classical and new variant strains must be accomplished through the molecular characterisation of a viral gene, usually the capsid encoding gene.

Interestingly, although macroscopic examination often indicated that lungs are more affected than liver ((Duarte et al., 2015a) *and this study*), the comparison of the Cq values obtained for these two organs in 9 wild rabbits showed differences ranging from 0.66 to 6.87, revealing consistently higher viral charges in the liver. Due to the lower Cq values found in a few samples, a mean Cq value of 12.88 ± 3.88, corresponding to 1.5×10^8 RNA molecules, was calculated per mg of liver. This estimation is about $10\times$ higher than the value previously estimated for RHDV2 infected rabbits with the same method (Duarte et al., 2015b). Although this deviation may suggest that higher viral loads can be obtained in adult rabbits during RHDV2 infection, it may also have resulted from dehydration of the cadavers before being collected by the hunters. Sampling bias regarding the age of the animals may have also influenced these results since only wild adult rabbits were used in the present study.

No co-infections with G5 strains, or any other RHDV genetic groups, and RHDV2 were detected. Although the number of strains analysed in this study is limited to 27, the fact that since July 2014 no other type but RHDV2 was detected at the National reference laboratory (INIAV) suggests that RHDV2 is rapidly replacing the G5 strains previously circulating in Azores. This rapid takeover of the RHDV strains by RHDV2 is similar to what has been reported in several European Countries where wild populations of rabbits are present (Le Gall-Recule et al., 2013).

The recurrent appearance of two novel A/G SNPs (930th and 1294th positions) and two C/T SNPs (1689th and 1704th positions) in the *vp60* sequence of all Azorean strains along with the phylogenetic analysis further confirmed that the Azorean-RHDV2 cluster separately within the European-RHDV2 group (Fig. 4).

Interestingly, all non-synonymous substitutions found in 942-nucleotide region analysed of the Azorean strains are located within the hypervariable domains of the capsid protein (Wang et al., 2013) The $Gly_{432,}$ until now only detected in the Azorean strains, is located within the hypervariable domain V6, whereas Gly_{307} and Ile_{349}, shared also by a few other non-Azorean strains, are localised in the V1 and V2 domains, respectively.

Despite the high similarity to European RHDV2 strains, no absolute identity was found between the partial *vp60* sequences from Azores and any sequence available publically at the moment. However, the Bayesian and ML phylogenetic analyses revealed a closer genetic relationship with two strains from Mértola

(subregion Baixo Alentejo) (KM115712-13) and three strains from the Algarve (KM115714-16), albeit none of these strains exhibit the Azorean-characteristic molecular markers. Therefore, a direct epidemiological link between the incursion of RHDV2 in Azores and other non-Azorean strains could not be established, raising questions regarding its origin. Furthermore, other strains from Barrancos (subregion Baixo Alentejo), Mora and Estremoz (subregion Alentejo Central) and Estoi (Algarve), also included in the analysis, did not show the same level of genetic proximity with the Azorean strains, as the five sequences from Mértola and the Algarve (Fig. 4A).

It is yet to be determined if the specific genetic characteristics of the Azorean strains were already present in the source of the first incursion. The time gap described in the past for the incursion of RHDV in Faial (1988) (Carvalho et al., 1994), S. Jorge (1989) (Carvalho et al., 1993) and St. Maria Island (1990) (Carvalho and Almeida, 1991) contrasts with the short interval between the first report of RHDV2 in the different islands.

The transmission of RDHV can take place by direct contact between infected and non-infected rabbits, but it can also be conveyed between rabbits by means of inanimate objects (fomites). Insects are known to act as mechanical vectors in the transmission of the virus (Barratt et al., 1998; Crosby and McLennan, 1996). *Spilopsyllus cuniculi* species were reported in Terceira, S. Miguel and Faial in 1994 (Carvalho et al., 1994) and later in Pico and S. Jorge (Libois et al., 1997). These fleas were able to transmit the disease in laboratorial conditions (Lenghaus et al., 1994) (McColl et al., 2002), and along with flies and mosquitoes, may act as mechanical vector in the propagation of the virus in the Azorean islands. However, since the outbreaks of RHDV2 in Azores took place in the cooler seasons when mosquitoes are less active, its role in the dissemination of RHDV2 may be questionable. On the other hand, increasing attention has been given to the role of rodents in the spreading of RHDV. It is known that micromammals such as *Mus spretus* and *Apodemus sylvaticus* are environmental preservers and potential transmitters of RHDV (Merchan et al., 2011). Several non-native rodent species are found in Azores namely the *Rattus norvegicus*, *Rattus rattus* and the *Mus musculus* (Collares-Pereira et al., 2000; Medeiros et al., 2008), which may play a role in the dispersal of the disease.

The outbreak in Faial succeeded the incursion of RHDV2 in other islands from the Central group, namely Graciosa, Terceira and S. Jorge. Faial is 39 km away from S. Jorge, being the closest neighbouring islands of the archipelago. The frequent maritime traffic between the islands may be related with the quick spread of the virus to Faial and more recently to both islands of the Eastern group (St. Maria and S. Miguel).

The independent clustering of the Azorean strains disclosed by our phylogenetic analyses, and the low genetic distances found, reinforces the idea that a unique introduction of RHDV2 may have taken place in the archipelago, during late 2014 (Duarte et al., 2015a) and suggests an epidemiological link between these strains.

Taking into account the wide range of possibilities for the incursion and dissemination of RHDV2 in Azores, the full understanding of the recent outbreaks in the archipelago will require the combined analysis of further molecular information and epidemiological data.

Acknowledgements

The authors would like to thank to Maria João Teixeira, Ricardino Ferreira, Rosário Ferreira and Durval Costa for excellent technical assistance. Part of this work was supported by a grant from the Fundação para a Ciência e Tecnologia (FCT) SFRH/79225/2011.

References

Abrantes, J., Lopes, A.M., Dalton, K.P., Melo, P., Correia, J.J., Ramada, M., Alves, P.C., Parra, F., Esteves, P.J., 2013. New variant of rabbit hemorrhagic disease virus, Portugal, 2012–2013. Emerg. Infect. Dis. 19, 1900–1902.

Abrantes, J., Lopes, A.M., Esteves, P.J., 2012. Complete genomic sequences of rabbit hemorrhagic disease virus G1 strains isolated in the European rabbit original range. J. Virol. 86, 13886.

Baily, J.L., Dagleish, M.P., Graham, M., Maley, M., Rocchi, M.S., 2014. RHDV variant 2 presence detected in Scotland. Vet. Rec. 174, 411.

Barratt, B.I.P., Ferguson, C.M., Heath, A.C.G., Evans, A.A., Logan, R.A.S., 1998. Can insects transmit rabbit haemorrhagic disease virus? In:. Proceedings of the 51st New Zealand Plant Protection Conference, pp. 245–250.

Bergin, I.L., Wise, A.G., Bolin, S.R., Mullaney, T.P., Kiupel, M., Maes, R.K., 2009. Novel calicivirus identified in rabbits, Michigan, USA. Emerg. Infect. Dis. 15, 1955–1962.

Capucci, L., Fusi, P., Lavazza, A., Pacciarini, M.L., Rossi, C., 1996. Detection and preliminary characterization of a new rabbit calicivirus related to rabbit hemorrhagic disease virus but nonpathogenic. J. Virol. 70, 8614–8623.

Carvalho, G.D.F., Almeida, L.M.M., 1991. Contribuição para o estudo de uma populacional de coelhos selvagens (*Oryctolagus cuniculus*, L.) na Ilha de Santa Maria e o impacto do RVHD na população local. Relatórios e Comunicações do Departamento de Biologia, Universidade dos Açores 19, 61–67.

Carvalho, G.D.F., Ferrand, N., Fonseca, A., Branco, M., Azevedo, M., Mendes, R., Batista, P., Mântua, P., 1993. Estudo de uma população de coelhos selvagens (*Oryctolagus cuniculus*, L.) na Ilha de São Jorge, Açores. Relatórios e Comunicações do Departamento de Biologia, Universidade dos Açores 21, 8–20.

Carvalho, G.D.F., Fonseca, A., Cruz, A., Célio, P., Mântua, P., Simões, C., Silva, S., Arruda, G., 1994. Estudo Preliminar de alguns parâmetros de uma população de coelho selvagem (*Oryctolagus cuniculus*) da Ilha do Faial, Açores. Relatórios e Comunicações do Departamento de Biologia, Universidade dos Açores 22, 49–60.

Collares-Pereira, M., Mathias, M.L., Santos-Reis, M., Ramalhinho, M.G., Duarte-Rodrigues, P., 2000. Rodents and Leptospira transmission risk in Terceira island (Azores). Eur. J. Epidemiol. 16, 1151–1157.

Cook, H.C., 1997. Origins of ... tinctorial methods in histology. J. Clin. Pathol. 50, 716–720.

Crosby, T.K., McLennan, J., 1996. Potential vectors of rabbit calicivirus disease (RCD) in New Zealand: a review. MAF Policy, pp 29.

Dalton, K.P., Nicieza, I., Balseiro, A., Muguerza, M.A., Rosell, J.M., Casais, R., Alvarez, A.L., Parra, F., 2012. Variant rabbit hemorrhagic disease virus in young rabbits, Spain. Emerg. Infect. Dis. 18, 2009–2012.

Duarte, M., Henriques, M., Barros, S.C., Fagulha, T., Ramos, F., Luis, T., Fevereiro, M., Benevides, S., Flor, L., Barros, S.V., Bernardo, S., 2015a. Detection of RHDV variant 2 in the Azores. Vet. Rec. 176, 130.

Duarte, M.D., Barros, S.C., Henriques, A.M., Fagulha, M.T., Ramos, F., Luis, T., Fevereiro, M., 2014a. Development and validation of a real time PCR for the detection of myxoma virus based on the diploid gene M000.5L/R. J. Virol. Methods 196, 219–224.

Duarte, M.D., Carvalho, C.L., Barros, S.C., Henriques, A.M., Ramos, F., Fagulha, T., Luis, T., Duarte, E.L., Fevereiro, M., 2015b. A real time Taqman RT-PCR for the detection of rabbit hemorrhagic disease virus 2 (RHDV2). J. Virol. Methods 219, 90–95.

Duarte, M.D., Henriques, A.M., Barros, S., Luis, T., Fagulha, T., Ramos, F., Fevereiro, M., 2014b. New insight into the epidemiology of rabbit hemorrhagic disease viruses in Portugal: retrospective study reveals the circulation of genogroup 5 (G5) in Azores and discloses the circulation of G1 and G6 strains in mainland until 2008. Infect. Genet. Evol. 27, 149–155.

Forrester, N.L., Trout, R.C., Gould, E.A., 2007. Benign circulation of rabbit haemorrhagic disease virus on Lambay Island, Eire. Virology 358, 18–22.

Felsenstein, J., 2004. Inferring Phylogenies. Sinauer Associates, Sunderland, Massachusetts.

Guindon, S., Dufayard, J.F., Lefort, V., Anisimova, M., Hordijk, W., Gascuel, O., 2010. New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. Syst. Biol. 59, 307–321.

Hasegawa, M., Kishino, H., Yano, T., 1985. Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. J. Mol. Evol. 22, 160–174.

Huelsenbeck, J.P., Ronquist, F., Nielsen, R., Bollback, J.P., 2001. Bayesian inference of phylogeny and its impact on evolutionary biology. Science 294, 2310–2314.

Jahnke, M., Holmes, E.C., Kerr, P.J., Wright, J.D., Strive, T., 2010. Evolution and phylogeography of the nonpathogenic calicivirus RCV-A1 in wild rabbits in Australia. J. Virol. 84, 12397–12404.

Lavazza, A., Scicluna, M.T., Capucci, L., 1996. Susceptibility of hares and rabbits to the European brown hare syndrome virus (EBHSV) and rabbit haemorrhagic disease virus (RHDV) under experimental conditions. Zentralblatt fur Veterinarmedizin. Reihe B. J. Vet. Med. Series B 43, 401–410.

Le Gall-Recule, G., Lavazza, A., Marchandeau, S., Bertagnoli, S., Zwingelstein, F., Cavadini, P., Martinelli, N., Lombardi, G., Guerin, J.L., Lemaitre, E., Decors, A., Boucher, S., Le Normand, B., Capucci, L., 2013. Emergence of a new lagovirus related to Rabbit Haemorrhagic Disease Virus. Vet. Res. 44, 81.

Le Gall-Recule, G., Zwingelstein, F., Boucher, S., Le Normand, B., Plassiart, G., Portejoie, Y., Decors, A., Bertagnoli, S., Guerin, J.L., Marchandeau, S., 2011a. Detection of a new variant of rabbit haemorrhagic disease virus in France. Vet. Rec. 168, 137–138.

Le Gall-Recule, G., Zwingelstein, F., Fages, M.P., Bertagnoli, S., Gelfi, J., Aubineau, J., Roobrouck, A., Botti, G., Lavazza, A., Marchandeau, S., 2011b. Characterisation of a non-pathogenic and non-protective infectious rabbit lagovirus related to RHDV. Virology 410, 395–402.

Le Gall-Recule, G., Zwingelstein, F., Laurent, S., de Boisseson, C., Portejoie, Y., Rasschaert, D., 2003. Phylogenetic analysis of rabbit haemorrhagic disease virus in France between 1993 and 2000, and the characterisation of RHDV antigenic variants. Arch. Virol. 148, 65–81.

Lenghaus, C., Westbury, H.A., Collins, B.J., Ratnamohan, N., Morrissy, C., 1994. Overview of the RHD project in Australia. In: Munro R.K., W.R. (Ed.), Rabbit Haemorrhagic Disease: Issues in Assessment for Biological Control. Australian Government Publishing Service, Canberra, pp. 104–129.

Libois, R., Fons, F., Mathias, M.-L., Santos-Reis, M., Petrucci-Fonseca, F., Ramalhinho, M.G., Oom, M.M., 1997. Notes on the flea fauna (Insecta, Siphonaptera) of the terrestrial Azorean mammals. Arquivos do Museu Bocage 3, 1–11.

Lopes, A.M., Correia, J., Abrantes, J., Melo, P., Ramada, M., Magalhaes, M.J., Alves, P.C., Esteves, P.J., 2015. Is the new variant RHDV replacing genogroup 1 in Portuguese wild rabbit populations? Viruses 7, 27–36.

Lui, S.J., Xue, H.P., Pu, B.Q., Qian, N.H., 1984. A new viral disease of rabbits. Animal Husbandry Vet. Med. 16, 253–255.

McColl, K.A., Merchant, J.C., Hardy, J., Cooke, B.D., Robinson, A., Westbury, H.A., 2002. Evidence for insect transmission of rabbit haemorrhagic disease virus. Epidemiol. Infect. 129, 655–663.

McIntosh, M.T., Behan, S.C., Mohamed, F.M., Lu, Z., Moran, K.E., Burrage, T.G., Neilan, J.G., Ward, G.B., Botti, G., Capucci, L., Metwally, S.A., 2007. A pandemic strain of calicivirus threatens rabbit industries in the Americas. Virol. J. 4, 96.

Medeiros, F., Fonseca, A., Gouveia, C., Nunes, R., Vieira, J., Veiga, M., Nóia, M., Fraga, M., 2008. Conservação dos vertebrados terrestres das flores e do corvo. XIII expedição científica do Departamento de Biologia" / Universidade dos Açores, pp. 49–58.

Merchan, T., Rocha, G., Alda, F., Silva, E., Thompson, G., de Trucios, S.H., Pages, A., 2011. Detection of rabbit haemorrhagic disease virus (RHDV) in nonspecific vertebrate hosts sympatric to the European wild rabbit (*Oryctolagus cuniculus*). Infect. Genet. Evol. 11, 1469–1474.

Moss, S.R., Turner, S.L., Trout, R.C., White, P.J., Hudson, P.J., Desai, A., Armesto, M., Forrester, N.L., Gould, E.A., 2002. Molecular epidemiology of Rabbit haemorrhagic disease virus. J. Gen. Virol. 83, 2461–2467.

Muller, A., Freitas, J., Silva, E., Le Gall-Recule, G., Zwingelstein, F., Abrantes, J., Esteves, P.J., Alves, P.C., van der Loo, W., Kolodziejek, J., Nowotny, N., Thompson, G., 2009. Evolution of rabbit haemorrhagic disease virus (RHDV) in the European rabbit (*Oryctolagus cuniculus*) from the Iberian Peninsula. Vet. Microbiol. 135, 368–373.

Nicholas, K.B., Nicholas, H.B.J., Deerfield, D.W.I., 1997. GeneDoc: Analysis and Visualization of Genetic Variation. EMBNEW.NEWS 4.

Puggioni, G., Cavadini, P., Maestrale, C., Scivoli, R., Botti, G., Ligios, C., Le Gall-Recule, G., Lavazza, A., Capucci, L., 2013. The new French 2010 Rabbit Hemorrhagic Disease Virus causes an RHD-like disease in the Sardinian Cape hare (*Lepus capensis mediterraneus*). Vet. Res. 44, 96.

Ronquist, F., Huelsenbeck, J.P., 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19, 1572–1574.

Tavaré, S., 1986. Some probabilistic and statistical problems in the analysis of DNA sequences. In: Miura, R.M. (Ed.), Lectures on Mathematics in the Life Sciences, 17. American Mathematical Society, Providence (RI), pp. 57–86.

Tham, K.M., Barnes, S.M., Hunter, S.N., 1999. Polymerase chain reaction amplification and gene sequence analysis of a calicivirus from a feral rabbit. Virus Genes 18, 235–242.

Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. Clustal-W – improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673–4680.

Wang, X., Xu, F., Liu, J., Gao, B., Liu, Y., Zhai, Y., Ma, J., Zhang, K., Baker, T.S., Schulten, K., Zheng, D., Pang, H., Sun, F., 2013. Atomic model of rabbit hemorrhagic disease virus by cryo-electron microscopy and crystallography. PLoS Pathog. 9, e1003132.

Waterhouse, A.M., Procter, J.B., Martin, D.M., Clamp, M., Barton, G.J., 2009. Jalview Version 2-a multiple sequence alignment editor and analysis workbench. Bioinformatics 25, 1189–1191.

Westcott, D.G., Frossard, J.P., Everest, D., Dastjerdi, A., Duff, J.P., Steinbach, F., Choudhury, B., 2014. Incursion of RHDV2-like variant in Great Britain. Vet. Rec. 174, 333.

Study 9

Are the Madeira archipelago RHDV2 viruses distinct from those characterised so far?

Emergence of rabbit haemorrhagic disease virus 2 in the archipelago of Madeira, Portugal (2016-2017).

Carina L. Carvalho[1], Sara Silva[2], Paz Gouveia[3], Margarida Costa[3], Elsa L. Duarte[1,4], Ana M. Henriques[5], Sílvia S. Barros[5], Tiago Luís[5], Fernanda Ramos[5], Teresa Fagulha[5], Miguel Fevereiro[5], Margarida D. Duarte[5]

[1]Instituto de Ciências Agrárias e Ambientais Mediterrânicas (ICAAM) and Instituto de Investigação e Formação Avançada (IIFA), Universidade de Évora, Évora, Portugal; [2]Direção Regional para a Administração Pública do Porto Santo (DRAPS), Porto Santo, Portugal; [3]Laboratório Regional de Veterinária e Segurança Alimentar, Direção Regional de Agricultura (DRA) da Região Autónoma da Madeira, Madeira, Portugal; [4]Departamento de Medicina Veterinária, Universidade de Évora, Évora, Portugal; [5]Instituto Nacional de Investigação Agrária e Veterinária (INIAV), Lisboa, Portugal;

Virus Genes doi: 10.1007/s11262-017-1483-6. [Epub ahead of print]

Virus Genes
DOI 10.1007/s11262-017-1483-6

Emergence of rabbit haemorrhagic disease virus 2 in the archipelago of Madeira, Portugal (2016–2017)

Carina Luísa Carvalho[1,2] · Sara Silva[3] · Paz Gouveia[4] · Margarida Costa[4] · Elsa Leclerc Duarte[1,2,5] · Ana Margarida Henriques[6] · Sílvia Santos Barros[6] · Tiago Luís[6] · Fernanda Ramos[6] · Teresa Fagulha[6] · Miguel Fevereiro[6] · Margarida Dias Duarte[6]

Received: 4 April 2017 / Accepted: 15 June 2017
© Springer Science+Business Media, LLC 2017

Abstract We report the detection of rabbit haemorrhagic disease virus 2 (RHDV2) in the Madeira archipelago, Portugal. Viral circulation was confirmed by RT-qPCR and *vp60* sequencing. Epidemiological data revealed the outbreak initiated in October 2016 in Porto Santo affecting wild and domestic rabbits. It was then detected three months later on the island of Madeira. Five haplotypes were identified and a genetic overall similarity of 99.54 to 99.89% was observed between the two viral populations. Unique single nucleotide polymorphisms were recognised in the Madeira archipelago strains, two of which resulting in amino acid substitutions at positions 480 and 570 in the VP60 protein. Phylogenetic investigation by Maximum Likelihood showed all the *vp60* sequences from the Madeira archipelago group together with high bootstraps. The analysis also showed that the Madeira archipelago strains are closely related to the strains detected in the south of mainland Portugal in 2016, suggesting a possible introduction from the mainland. The epidemiological data and high genetic similarity indicate a common source for the Porto Santo and Madeira RHDV2 outbreaks. Human activity related to hunting was most probably at the origin of the Madeira outbreak.

Keywords Rabbit haemorrhagic disease (RHDV) · Wild rabbits · Domestic rabbits · Madeira archipelago · Macaronesian region

Text body

Rabbit haemorrhagic disease virus 2 (RHDV2), a small fast-evolving RNA virus (mean 5.48 to 7.7 $\times$ 10^{-4} substitutions/site/year [1, 2]) belonging to the *Caliciviridae* family [3], was first detected in Europe in 2010 [4]. Like the genetically related RHDV that emerged in 1984 [5], it causes an often fatal haemorrhagic disease (RHD) in European rabbits (*Oryctolagus cuniculus*) [4, 6].

Madeira Island was the first region of the Portuguese territory to register RHD following the importation of contaminated rabbit meat from China in 1987 (reviewed by [7]). The Madeira archipelago is situated in the North Atlantic Ocean, 1040 km southwest of mainland Portugal, 490 km from the Canary Islands and about 935 km from

Edited by William Dundon.

Electronic supplementary material The online version of this article (doi:10.1007/s11262-017-1483-6) contains supplementary material, which is available to authorized users.

✉ Margarida Dias Duarte
margarida.duarte@iniav.pt

1 Instituto de Ciências Agrárias e Ambientais Mediterrânicas (ICAAM), Universidade de Évora, Núcleo da Mitra, 7000, Évora, Portugal

2 Instituto de Investigação e Formação Avançada (IIFA), Universidade de Évora, Núcleo da Mitra, 7000, Évora, Portugal

3 Direção Regional para a Administração Pública do Porto Santo (DRAPS), Avenida Vieira de Castro, n°1, 9400-179 Porto Santo, Portugal

4 Laboratório Regional de Veterinária e Segurança Alimentar, Direção Regional de Agricultura (DRA) da Região Autónoma da Madeira, Caminho das Quebradas de Baixo, n° 79, S. Martinho, 9000-233 Funchal, Madeira, Portugal

5 Departamento de Medicina Veterinária, Universidade de Évora, Núcleo da Mitra, 7000, Évora, Portugal

6 Laboratório de Virologia, Instituto Nacional de Investigação Agrária e Veterinária (INIAV), Av. da República, Quinta do Marquês, 2780-157 Oeiras, Portugal

Published online: 21 June 2017

the Moroccan coast. This archipelago includes the Madeira, Porto Santo and Desertas islands.

The wild rabbit (*Oryctolagus cuniculus algirus*) was introduced into the archipelago probably from South-western Portugal during the 15th century by Portuguese navigators, [8]. According to the official veterinary services the size of the wild populations in Porto Santo and Madeira was never estimated, and no census is currently available for the domestic populations. There is no industrial farming in the archipelago, and local backyard rabbitries (for private consumption) contained no more than 10 and 25 rabbits in Porto Santo and Madeira, respectively.

RHDV circulated in Madeira and Porto Santo until at least 2011 and late 2012, respectively (Duarte MD and Costa M, *unpublished data*). It was subsequently controlled through vaccination.

At the end of 2016, an increase in mortality was registered in the wild rabbits on Porto Santo, and three months later on Madeira. Field investigation revealed that the first casualties occurred in October amongst the wild rabbit population from the central region of Porto Santo (Campo de Cima and Pico do Castelo, 3.5 km apart). In the following weeks, mortality was also seen in other areas, (Dragoal) but this time also affecting domestic rabbits. Three months later, in January 2017, four wild rabbits were found dead in Paul da Serra, the biggest and highest mountainous plateau in Madeira (municipality of Ponta do Sol). At the time, both a farm and pet rabbit (the latter vaccinated twice against myxomatosis and classical RHDV) from the municipalities of Santa Cruz and Funchal, respectively, were also received at the Regional Laboratory of Madeira (Funchal) and sent to the Reference Laboratory for Animal Diseases (INIAV) in Oeiras for diagnosis.

In this study, we analysed ten rabbits (seven wild and three domestic) originating from both islands. Necropsies were carried out at the Regional Laboratory of Madeira. All specimens presented with congestion and haemorrhage of the tracheal mucosa and lungs, confirmed by histopathology performed by standard procedures [9]. In half of the animals ($n = 5$), the liver was marbled and presented mild discoloration. Renal congestion was observed in most of the animals ($n = 8$). The microscopic alterations were compatible with systemic coagulopathy and multi-organ failure. Several degenerative and necrotic signs, namely vacuolar degeneration, karyorrhexis and pyknotic nuclei, were observed in the liver and kidneys of most specimens. Virological examination was carried out at INIAV. The presence of RHDV2 was confirmed in the liver samples of all rabbits by molecular methods [10]. RHDV2 had not previously been reported in the archipelago, despite known circulation on the mainland since 2012 [11], and on the Azores [12] and Tenerife [13] since 2015. All samples tested negative for the myxoma virus (MYXV-DNA) by the method described by Duarte et al. [14]. Sequencing of the PCR product was obtained with primers RHD-9F and RHD-10R [15], which amplify both RHDV and RHDV2, revealed that RHDV2, and not classical RHDV strains, caused the death of the ten rabbits.

The complete *vp60* sequences from seven liver samples, three from Porto Santo and four from Madeira, were obtained using the pairs of primers 27F [16] and 986R [17] and 717F [17] and 10R [15] and submitted to GenBank (accession numbers KY310747-KY310749 and KY783700-KY783703). Two strains from Porto Santo were identical (KY310748, KY310749), differing only in one residue from the third (KY310747) (similarity of 99.94–100%). Two strains from Madeira were also found to be identical (KY783702, KY783703), but diverged from the other two by five (KY783700) and eight residues (KY783701), respectively, corresponding to a genetic similarity of 99.43–100%.

Seven single nucleotide polymorphisms (SNPs) were identified in the Madeira archipelago 2016–2017 strains (supplementary material, Table 1). Of these, two were non-synonymous encoding Asn_{480} ($n = 6$, 85.7%) or Ser_{480} ($n = 1$, 14.3%) and Glu_{570}, ($n = 7$, 100%). None of these amino acids have been detected before in this position, given that a Thr_{480}, and less frequently an Ala_{480}, are usually found. Similarly, in position 570 only a Gly_{570} had been identified to date.

Additionally, six synonymous and two non-synonymous (encoding residues 347 and 369) SNPs were also found in the viruses from the Madeira archipelago, only shared by a few other strains (supplementary material, Table 1). Altogether, the seven specific and eight rare SNPs defined a DNA fingerprint for the RHDV2 2016–2017 strains from the archipelago.

Residues Ala_{347}, Tyr_{369} and Asn_{480}/Ser_{480}, are located in the P2 sub-domain of the VP60 protein, responsible for virus–host receptor interactions and antigenic diversity [18–21]. Residue 480 falls within the V7 hypervariable region, one of the regions of the P2 sub-domain where variation among rabbit caliciviruses is higher [22]. Residue Glu_{570} is located in the P1 sub-domain of VP60 [22].

Residues 570 and 480 lay in loops not spatially constrained within the P1 and P2 sub-domains. However, given the different chemical properties and sizes of the replacing aa, their potential impact on the 2D-structure (SS) was explored. The SS of sequences KY310747-48 and KY783700-02 was compared with that of strain FR81978, using the molecule c3zueB as a model (Phyre2 software) [23]. The α-helix predicted at position 492–499 in sequence FR819781 (Ala_{480}) was extended upstream by two residues in sequence KY783701 (Ser_{480}). In the remaining strains, the Asn_{480} appears to have abrogated the α-helix, instead folding a β-strand (residues 484–498),

which, if present, did not compromise the virulence or pathogenicity.

Maximum Likelihood (ML) phylogeny based on the complete *vp60* sequences revealed that the Porto Santo and Madeira viruses cluster together with a bootstrap value of 100 (Fig. 1a), confirming their higher genetic similarities (99.54–99.89%), low genetic distances, low average number of nucleotide differences and substitutions (3.667, supplementary material Table 2), and the null number of fixed differences between these populations (supplementary material Table 2). Since 2012, there are no records of legal live rabbit trading into Porto Santo or Madeira and rabbit meat is received mainly from the mainland, although it is sometimes imported from a few European Union (EU) Member States (France and Spain). Entrance of the virus through contaminated meat or rabbit by-products is therefore highly unlikely due to sanitary control. However, the intense human movement between the Madeira archipelago and the Portuguese mainland constitute a risk factor for virus introduction. The short time gap between the two outbreaks suggests that the virus entered the archipelago through Porto Santo from where it disseminated to

Fig. 1 **a, b** Maximum Likelihood (ML) phylogenetic trees based on **(a)** complete *vp60* sequences (1740 nt) and **(b)** 641-nt long concatenate sequences from RHDV2 strains from the Madeira and Azores archipelagos, other mainland regions and other countries, available in GenBank. The appropriated substitution models were determined resourcing to R software (R Development Core Team, 2009). The general time reversible (GTR) model [26] with a **(a)** discrete gamma distribution (+G) and/or an allowance for the incorporation of invariant sites (+I) (GTR + G+I) and (b) a gamma-distributed rate variation across sites (GTR + G) showed the lowest BIC and AICc values and were used to infer the phylogenetic relationships. Robustness of the tree nodes was assessed by bootstrapping 1000 times. The graphical representations and edition of the phylogenetic trees were performed with FigTree v1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/). Only bootstrap (BS) values equal or greater than 70 are shown. Sequence KC345614 (not displayed) was chosen as outgroup to root the trees. In both trees each *letter* represents a particular sequence or a group of sequences whose detailed information is shown in supplementary Table 3. With exception of groups l, m, n, o and p, which are pointed in both trees, the country/region of origin and year of collection is highlighted only in the concatenated sequences-based tree (b). **c** Geographic map with the relative location of the Madeira (*red*), Azores (*green*) and Tenerife (*purple*) archipelagos with relation to mainland Portugal (Western Iberia) and Africa. The location of Barrancos is shown by a *black circle* in the mainland territory. To allow comparison, the same colour code for strains is used in both trees. **d** Close-up of the Madeira archipelago strains with the accession numbers of the sequences

Madeira. On Madeira and Porto Santo, wild rabbits are small game species and hunting represents a significant local recreational activity. Porto Santo is a popular destination for this activity and hunters from Madeira generally take their hunting trophies back home, a high-risk practice that may have led to the island's outbreak three months after RHDV2 emerged in Porto Santo.

Our study suggests that the Madeira archipelago viruses may have originated from mainland Portugal, or share a recent common ancestor with the strains identified in the southern Alentejo region (NUT II PT 18) in 2016 (Fig. 1a, b). This relationship is also evidenced by the genetic data (supplementary material, Tables 1 and 2). However, the lack of detailed classical epidemiologic information and the relatively high average number of nucleotide differences (34.333 and 37.000, supplementary material Table 2) found between the Barrancos 2016 and Madeira archipelago virus populations hampers the establishment of a confident direct link between the two outbreaks.

Since RHDV2 was registered in Tenerife in 2015 [13], and given its relative proximity with the Madeira archipelago, introduction from the Canaries was also considered. This possibility was discarded after assessment through a ML-tree based on concatenated *vp60* sequences, constructed as described by Martin-Alonzo et al. [13] as only partial *vp60* gene sequences from Tenerife were available. The Tenerife sequences grouped together in a different cluster, distant from the Madeira archipelago group. They appear to be more closely related to RHDV2 strains found in Spain (KP129395) and in the north of Portugal (KM979445) in 2012, or even with strains from south and central mainland Portugal (found between 2013 and 2015) (Fig. 1b), than with the Madeira or Barrancos 2016 strains. Furthermore, none of the amino acids Asn_{480}/Ser_{480} and Glu_{570}, the genetic markers of the Madeira strains, were present in the Tenerife strains.

The identification of two identical strains (KY310748 and KY310749) in both wild and domestic rabbits from Porto Santo, found dead within a short timeframe, provides evidence on the rapid transmission between these populations.

New RHDV2 outbreaks continue to be reported in Europe (e.g. Norway, Sweden, Finland) [24], and in Africa (Tunisia) [24] and Australia [25]. Seven years after its first detection, RHDV2 deeply affected the three archipelagos of the Macaronesia, demonstrating the rapid dissemination of the virus beyond continental Europe and raising concerns regarding the spread of the virus to nearby African countries.

Acknowledgements The authors thank Maria João Teixeira, Fátima Cordeiro, Ricardino Ferreira and Maria da Graça Lemos Gomes for their technical assistance. This study was partially funded by a Grant and a project from the Fundação para a Ciência e Tecnologia (FCT) (SFRH/79225/2011 and Project UID/AGR/00115/2013).

Author contributions Conceived of or Designed study: MDD, CLC, ELD, MF; Performed research: CLC, MC, SS, PG, MDD; Analysed data: CLC, AMH, SSB, MDD, FR, TF, TL; Wrote paper: MDD, CLC.

Compliance with ethical standards

Conflict of interest The author declares that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

References

1. P.J. Kerr, A. Kitchen, E.C. Holmes, J. Virol. **83**, 12129 (2009)
2. F. Alda, T. Gaitero, M. Suárez, T. Merchán, G. Rocha, I. Doadrio, BMC Evol. Biol. **10**, 347 (2010)
3. K.P. Dalton, J. Abrantes, A.M. Lopes, I. Nicieza, A.L. Alvarez, P.J. Esteves, F. Parra, Arch. Virol. **160**, 877 (2015)
4. G. Le Gall-Reculé, F. Zwingelstein, S. Boucher, B. Le Normand, G. Plassiart, Y. Portejoie, A. Decors, S. Bertagnoli, J.-L. Guérin, S. Marchandeau, Vet. Rec. **168**, 137 (2011)
5. J. Liu, H.P. Xue, B.Q. Pu, N.H. Qian, Anim. Husb. Vet. Med. **16**, 253–255 (1984)
6. G. Le Gall-Reculé, A. Lavazza, S. Marchandeau, S. Bertagnoli, F. Zwingelstein, P. Cavadini, N. Martinelli, G. Lombardi, J.-L. Guérin, E. Lemaitre, A. Decors, S. Boucher, B. Le Normand, L. Capucci, Vet. Res. **44**, 81 (2013)
7. M.D. Duarte, A.M. Henriques, S. Barros, T. Luís, T. Fagulha, F. Ramos, M. Fevereiro, Infect. Genet. Evol. **27**, 149 (2014)
8. P.J. Esteves, Molecular and population genetic analysis of polymorphism at the antibody loci IgGCH2 and IgVH in lagomorphs. PhD Thesis, Faculdade de Ciências, Porto University, 2003.
9. H.C. Cook, J. Clin. Pathol. **50**, 716 (1997)
10. M.D. Duarte, C.L. Carvalho, S.C. Barros, A.M. Henriques, F. Ramos, T. Fagulha, T. Luís, E.L. Duarte, M. Fevereiro, J. Virol. Methods **219**, 90 (2015)
11. J. Abrantes, A.M. Lopes, K.P. Dalton, P. Melo, J.J. Correia, M. Ramada, P.C. Alves, F. Parra, P.J. Esteves, Emerg. Infect. Dis. **19**, 1900 (2013)
12. M. Duarte, M. Henriques, S.C. Barros, T. Fagulha, F. Ramos, T. Luís, M. Fevereiro, S. Benevides, L. Flor, S.V. Barros, S. Bernardo, Vet. Rec. **176**, 130 (2015)
13. A. Martin-Alonso, N. Martin-Carrillo, K. Garcia-Livia, B. Valladares, P. Foronda, Infect. Genet. Evol. **44**, 46 (2016)
14. M.D. Duarte, S.C. Barros, A.M. Henriques, M.T. Fagulha, F. Ramos, T. Luís, M. Fevereiro, J. Virol. Methods **196**, 219 (2014)
15. K.M. Tham, S.M. Barnes, S.N. Hunter, Virus Genes **18**, 235 (1999)
16. G. Le Gall, C. Arnauld, E. Boilletot, J.P. Morisse, D. Rasschaert, J. Gen. Virol. **79**, 11–16 (1998)
17. M. Duarte, C. Carvalho, S. Bernardo, S.V. Barros, S. Benevides, L. Flor, M. Monteiro, I. Marques, M. Henriques, S.C. Barros, T. Fagulha, F. Ramos, T. Luís, M. Fevereiro, Infect. Genet. Evol. **35**, 163 (2015)
18. J. Bárcena, N. Verdaguer, R. Roca, M. Morales, I. Angulo, C. Risco, J.L. Carrascosa, J.M. Torres, J.R. Castón, Virology **322**, 118 (2004)

19. Z. Hu, X. Tian, Y. Zhai, W. Xu, D. Zheng, F. Sun, Protein Cell **1**, 48 (2010)
20. D. Luque, J.M. González, J. Gómez-Blanco, R. Marabini, J. Chichón, I. Mena, I. Angulo, J.L. Carrascosa, N. Verdaguer, B.L. Trus, J. Bárcena, J.R. Castón, J. Virol. **86**, 6470 (2012)
21. J. Bárcena, B. Guerra, I. Angulo, J. González, F. Valcárcel, C.P. Mata, J.R. Castón, E. Blanco, A. Alejo, Vet. Res. **46**, 106 (2015)
22. X. Wang, F. Xu, J. Liu, B. Gao, Y. Liu, Y. Zhai, J. Ma, K. Zhang, T.S. Baker, K. Schulten, D. Zheng, H. Pang, F. Sun, PLoS Pathog. **9**, e1003132 (2013)
23. L.A. Kelley et al., Nat. Protoc. **10**, 845–858 (2015)
24. World Organisation for Animal Health (OIE) Technical disease cards. 2016. Rabbit Haemorrhagic Disese. Aetology, Epidemiology, Diagnosis, Prevention and Control References
25. R.N. Hall, J.E. Mahar, S. Haboury, V. Stevens, E.C. Holmes, T. Strive, Emerg. Infect. Dis. **21**, 2276 (2015)
26. S. Tavaré, Some probabilistic and statistical problems in the analysis of DNA sequences, in *Lectures On Math- ematics in the Life Sciences*, vol. 17, ed. by R.M. Miura (American Mathematical Society, Providence (RI), 1986), pp. 57–86

Chapter VI

Tularaemia laboratorial diagnosis and genotyping methods

1. Update on the laboratory diagnosis of tularaemia and genotyping methods

1.1. Culture

F. tularensis is a fastidious organism and the most suitable medium for the cultivation of this pathogen are reviewed in General Introduction.

The rapid cultivation of *F. tularensis* is important for detection and monitoring during natural outbreak events or intentional bioterrorism attacks (Morris et al., 2017).

Recently, regarding liquid growth media for detection of *F. tularensis*, the Pavlovich's medium T (a transparent nutrient medium T (tularemic) for cultivation of *F. tularensis* based on heart-brain infusion, yeast extract, bactotryptone, technical casamino acids, bactoagar, salts, L-cysteine and glucose plus polymyxin B (100 units/ml) developed by (Pavlovich and Mishan'kin, 1987)), was successfully re-evaluated as an optimal liquid medium suitable for enrichment of fastidious and/or highly pathogenic bacteria (Becker et al., 2016). The medium provides the use of wider temperature ranges for *F. tularensis* culture and, due to its transparency, can be used for the identification of strains using certain biochemical features as well as in studies with field materials (dead rodents, water)(Pavlovich and Mishánkin, 1987). Even more recently, Morris et al., (2017) developed a liquid growth medium formulation, using readily available supplements, capable of producing enhanced early growth of *F. tularensis*. This growth medium consisted in brain heart infusion (BHI) broth supplemented with 2% Vitox, 10% Fildes and 1% Histidine (BVFH) and promoted enhanced growth *F. tularensis* Type A.I, A.II and Type B strains, compared to BHI alone (Morris et al., 2017).

1.2 Serology

Recently, Eremkin et al. (2016) developed an immune enzyme and immunochromatographic test-systems for detecting *F. tularensis* based on a selected set of monoclonal antibodies with immunochemical activity to *F. tularensis* antigens. The method demonstrated good sensitivity and specificity.

In addition, Spehar-Délèze et al. (2016) described an electrochemiluminescence (ECL) immunosensor for the rapid detection of *F. tularensis* using whole antibodies or antibody fragments as capture biomolecule. A sandwich immunoassay was used. Lipopolysaccharide (LPS) or the whole inactivated bacterial cell were used as targets, and the $Ru(bpy)_3^{(2+)}$-encapsulated silicate

nanoparticles were linked to the secondary antibody and used as ECL labels. The assay was performed in a fluidic chip housed in a custom-built black box incorporating electronics, optics and fluidics. The limit of detection for LPS was 0.4 ng/mL and for the whole inactivated bacterial cell was 70 and 45 bacteria/mL when the capturing molecule was the whole antibody and the antibody F(ab) fragment, respectively (Spehar-Délèze et al., 2016).

1.3. Molecular methods for diagnosis and genotyping

Although culture is the gold-standard, this procedure is not routinely performed once it requires special equipment and containment as well as experience (Lai et al., 2016). Hence, substantial research in the development of new diagnostic techniques for this pathogen has been carried out including a variety of molecular methods, most of which were already addressed in General Introduction. New developments in sequencing technologies, specifically next-generation sequencing (NGS) approaches now allow for rapid and less expensive sequencing of the genome of a wide range of pathogenic bacteria (Antwerpen et al., 2015). NGS allows deep sequencing and the generation of abundant DNA sequences and is a powerful technique for the reliable identification of pathogens in clinical specimens (Kuroda et al., 2012). Multi-locus sequence typing (MLST) (Maiden et al., 2013), MLST^{+} based on whole genome sequencing (WGS) (Antwerpen et al., 2015) and Genome-wide DNA microarray analysis (regional difference (RD) analysis) have been proposed to characterise *F. tularensis*. WGS has recently emerged as a rapid and cost-effective approach (Didelot et al., 2012) and is revolutionizing taxonomy, phylogeny, genomic diversity and population dynamics of several clinically relevant microorganisms (Antwerpen et al., 2015). It has provided an outstanding resource for comparative genome studies, allowing high-resolution snapshots of the genetic diversity found within a given species (Dempsey et al., 2006).

1.3.1. Multi-locus sequence typing (MLST) and the gene-by-gene approach

MLST uses alleles as the unit of comparison, rather than nucleotide sequences. Each allelic change is counted as a single genetic event, regardless of the number of nucleotide polymorphisms involved (Maiden et al., 2013). The MLST approach retains information at all *loci* and avoids the need to categorize which changes are recent point mutations and which are due to recombination. MLST records the sequences of allelic variants, and the data can be used for sequence-based analyses (Maiden et al., 2013). Traditional MLST has been successful in evaluating the epidemiology of some bacteria (Mellmann et al., 2011)(Cody et al., 2013) but fails to provide sufficient

discrimination power for resolving differences among single strains of monomorphic, low diversity, asexual pathogens such as *F. tularensis* (Antwerpen et al., 2015). A gene-by-gene ($MLST^{+}$) approach revealed to be superior to the current standard method, canSNP or MLVA (Antwerpen et al., 2015).

1.3.2. $MLST^{+}$ based on Whole Genome Sequencing (WGS)

$MLST^{+}$ based on whole genome was recently introduced by Antwerpen et al. (2015) as a high resolution genotyping approach for the characterisation of *F. tularensis* strains from outbreaks. The method uses $MLST^{+}$ and gene-by-gene comparison combined with WGS. Antwerpen et al. (2015) used the IonTorrent PGMand Illumina MiSeq (Illumina, San Diego, US) to sequence the reference strain *F. tularensis* subsp. *holarctica* LVS to exclude technology-bias of $MLST^{+}$ before samples were compared in a gene-by-gene approach ($MLST^{+}$). For clonal species, like *F. tularensis,* extending the genetic analysis beyond the core genome may be necessary and resolution of the WGS analysis might be increased by incorporating specific elements (including accessory genes, repetitive elements such as RNA coding sequences, insertion elements, VNTR and pseudo-genes or genes of the FIP), mandatory for following micro-evolutionary events.

1.3.3. Genome-wide DNA microarray analysis

(Broekhuijsen et al., 2003) developed a DNA microarray, based on 1.832 clones from a shotgun library used for sequencing of the highly virulent *F. tularensis* subsp. *tularensis* strain SCHU S4 that allowed a genome-wide analysis of other strains representing all four subspecies. The microarray analysis confirmed limited genetic variation within *F. tularensis*. However, eight regions of difference (RD) were identified that allow distinguishing moderately from highly virulent subspecies. One of these regions, RD1, allowed for the first time the development of *an F. tularensis*-specific PCR assay that discriminates each one of the four subspecies.

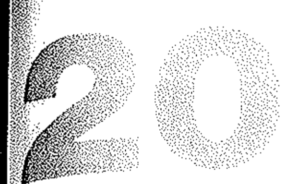
1.3.4. Other molecular methods

Recently, Banada et al. (2017) described a new cartridge-based assay with high sensitivity and specificity of 100%, for the rapid detection of *F. tularensis* in whole blood at the early stages of infection. The assay incorporates sample processing and detection into a single cartridge, making it suitable for rapid point-of-care detection. The assay limit of detection (LOD) and dynamic range were determined in a filter-based cartridge run on the GeneXpert system. The assay LOD was 0.1

genome equivalents (GE) per reaction and 10 CFU/ mL of *F. tularensis* in both human and macaque blood.

The use of real-time PCR for the detection of *F. tularensis* and discrimination between Type A and Type B is detailed next in the book chapter "Carvalho C.L., Duarte E.L., Zé-Zé L., Lopes de Carvalho I. 2014. Detecção de *Francisella tularensis* pela reacção de polimerse em cadeia em tempo real com sondas de hidrólise TaqMan. In *Abordagens moleculares em veterinária – como desvendar a etiologia e a epidemiologia da infecção*. Edições LIDEL 2014. ISBN: 978-989-752-034-1. pp. 271-276".

In addition, the Standard Method Performance Requirements (SMPRs) for Detection of *F. tularensis* in Aerosol Collection Devices (AOAC SMPR 2016.007) is given in Annex IV.



Deteção de *Francisella tularensis* pela reação de polimerase em cadeia em tempo real com sondas de hidrólise TaqMan®

Carina Luísa Carvalho, Elsa Leclerc Duarte, Líbia Zé-Zé, Isabel Lopes de Carvalho

SUMÁRIO

Francisella tularensis, agente etiológico de tularemia, é um agente zoonótico muito infecioso que se encontra classificado na lista de agentes de classe A de acordo com o *Centers for Disease Control and Prevention*. Na deteção de *F. tularensis*, a técnica de reação de polimerase em cadeia (PCR) em tempo real é preferencial, uma vez que apresenta elevada sensibilidade e especificidade. Esta metodologia pode ser usada numa grande variedade de amostras, incluindo as de origem humana, animal e ambiental. Neste capítulo, é descrita uma metodologia de PCR em tempo real para a deteção de *F. tularensis* e a discriminação das respetivas subespécies mais patogénicas, usando como alvos genómicos os *loci ISFtu2*, *tul4* e uma região divergente de uma ilha de patogenicidade denominada *Francisella pathogenicity island* (FPI).

INTRODUÇÃO

Francisella tularensis é o agente etiológico da tularemia. Atualmente é considerado como um dos microrganismos mais virulentos e com potencial uso num cenário de bioterrorismo, encontrando-se classificado como agente de classe A pelo *Centers for Disease Control and Prevention* (CDC)[1-3]. Este microrganismo pode causar doença em cerca de 150 espécies de vertebrados, incluindo lagomorfos e roedores, sendo relevante a sua transmissão ao Homem, quer por contacto direto, quer através dos vetores competentes como carraças e mosquitos. Estão descritas três subespécies de *F. tularensis* que podem ser encontradas em diferentes ambientes e regiões geográficas: *F. tularensis* subsp. *tularensis*, *F. tularensis* subsp. *holarctica* e *F. tularensis* subsp. *mediasiatica*[2,3]. Outra espécie, *F. novicida*, é considerada por muitos investigadores como uma quarta subespécie de *F. tularensis*, no entanto esta classificação não foi oficialmente adotada[3,4]. *F. tularensis* subsp. *tularensis* (Tipo A) é a mais patogénica e está associada a taxas de mortalidade elevadas no Homem. *F. tularensis* subsp. *holarctica* (Tipo B), apesar de patogénica, causa doença menos grave e raramente mortal[3,5-7]. *F. tularensis* subsp. *mediasiatica* ainda não foi associada a doença no Homem e *F. novicida* apresenta baixa patogenicidade para o Homem[3,5].

Os métodos moleculares baseados na PCR têm sido aplicados com sucesso na identificação rápida e na classificação de espécies de *Francisella*[1]. Estes métodos têm vindo a apresentar uma elevada sensibilidade e especificidade, constituindo ferramentas de diagnóstico importantes quando a cultura do agente, *gold standard* para o diagnóstico de tularemia, não é possível ou é negativa (ou na ausência de condições laboratoriais adequadas – laboratório de nível de segurança 3) e permitem reduzir os riscos de infeções adquiridas no laboratório[1,3,5,6].

© LIDEL – EDIÇÕES TÉCNICAS

Por outro lado, em virtude do seu potencial de utilização em bioterrorismo, a rápida deteção e identificação de *F. tularensis* é crítica em cenários de libertação intencional, sendo imprescindível na contenção de um eventual surto[2,8]. A técnica de PCR em tempo real permite detetar rapidamente a presença de ácidos nucleicos em concentrações mínimas, com pequenos volumes de reação e ciclos rápidos[8]. Esta metodologia foi desenvolvida para a deteção de *F. tularensis*, usando sondas de hidrólise.[5,9,10] A utilização de sondas marcadas com fluorocromos permite um aumento de sensibilidade numa magnitude de cerca de sete vezes comparativamente à PCR convencional. Adicionalmente, este tipo de sondas permite a visualização das curvas de amplificação em tempo real através da produção de um sinal fluorescente durante a reação de amplificação[5]. Esta técnica pode ser usada para detetar *F. tularensis* numa grande variedade de amostras, incluindo amostras ambientais e baseia-se na amplificação de várias sequências-alvo tais como: o elemento *ISFtu2*, uma sequência de inserção "element-*like*" presente em cópias múltiplas; e o gene *tul4*, que codifica para lipoproteínas da membrana externa de *F. tularensis*; entre outros alvos genómicos[5,9,10]. A amplificação em simultâneo de múltiplos alvos aumenta a especificidade da PCR, ao reduzir a probabilidade de ocorrência de falsos-positivos. O método baseado na amplificação do alvo genómico *ISFtu2* é o que apresenta maior sensibilidade e um limite de deteção de apenas uma célula, quer no caso de *F. tularensis* subsp. *tularensis* quer de *F. tularensis* subsp. *holarctica*, aumentando também a possibilidade da sua deteção em amostras ambientais, nas quais é expectável que o número de microrganismos seja mais baixo[5]. Este método já foi também aplicado com sucesso em amostras complexas, como tecidos de cadáveres de animais. Estes encontram-se geralmente muito contaminados por outras bactérias, dificultando o isolamento de *F. tularensis*, que é de crescimento fastidioso[5]. A técnica de PCR em tempo real foi também desenvolvida para a diferenciação entre subespécies na ausência de isolamento, nomeadamente entre *F. tularensis* subsp. *tularensis* (Tipo A) e *F. tularensis* subsp. *holarctica* (Tipo B)[7,9]. A distinção entre Tipo A e Tipo B é realizada recorrendo a um protocolo de PCR em tempo real, com sondas TaqMan®, direcionado para uma FPI que se verificou ser essencial para o crescimento de *F. tularensis* no interior dos macrófagos e para a virulência em ratinhos, apresentando diferenças nas duas subespécies. O protocolo para a deteção do Tipo A tem como alvo o gene *pdp*D, um gene de virulência que está presente no Tipo A e praticamente ausente no Tipo B, sugerindo a sua importância na patogenicidade[7,11]. O protocolo para deteção do Tipo B tem como alvo uma região flanqueante à posição do gene *pdpD* nos genomas Tipo A, entre o elemento de inserção *ISFtu2* (5') e a região de flanqueamento (3') (GenBank AY062040)[7].

Para tipificação das subespécies e para estudos epidemiológicos, têm sido aplicadas técnicas baseadas na amplificação de sequências genómicas repetidas em *tandem*[4,6,11], enquanto, para estudos filogenéticos, são recomendados marcadores mais discriminatórios, tais como polimorfismos nucleotídicos simples (SNP)[4].

Neste capítulo, apresentam-se os protocolos de PCR em tempo real, padronizados para o equipamento LightCycler®, para a deteção de *F. tularensis* e identificação das subespécies com importância clínica: *F. tularensis* subsp. *tularensis* e *F. tularensis* subsp. *holarctica*, usando como alvos genómicos os *loci ISFtu2*, *tul4* e uma região divergente da FPI[5,7,11].

1. MATERIAL

- Amostras a testar (com extração de DNA preferencialmente recorrendo a sistemas comerciais com coluna ou extratores ou automáticos; as amostras são manipuladas em laboratório de

© LIDEL – EDIÇÕES TÉCNICAS

biossegurança de nível 3 até à adição do tampão de lise).

- Controlo positivo: DNA de estirpes de referência de *F. tularensis* subsp. *tularensis* (por exemplo, SchuS4) e *F. tularensis* subsp. *holarctica* (por exemplo, LVS).
- Sondas TaqMan® e respetivos oligonucleótidos iniciadores (*primers*) flanqueadores específicos (Tabela 20.1).
- Reagentes para as reações de amplificação. Nos protocolos descritos neste capítulo são usados conjuntos de reagentes comercialmente disponíveis a partir do fabricante do equipamento LightCycler® (*Roche Applied Science*), contendo água, $MgCl_2$ (25 mM) e a mistura de PCR que contém a polimerase de DNA, o tampão da enzima e desoxirribonucleótidos trifosfatados (dNTP). No entanto, os protocolos podem ser adaptados para uso noutros equipamentos de PCR em tempo real.
- Outros consumíveis e utensílios para utilização com o equipamento LightCycler®: capilares de vidro, onde ocorrem as reações de amplificação (volume de 20 μL) e caneta para selar os capilares contendo os componentes da reação.
- Equipamento LightCycler® 2.0 (*Roche Applied Science*). Este equipamento possibilita realizar reações rápidas de PCR com deteção em tempo real, quantificar o ácido nucleico alvo e analisá-lo posteriormente à reação de amplificação.

2. PROCEDIMENTO OPERACIONAL

2.1. PCR em tempo real para os alvos *tul4* e *ISFtu2*

1. Preparar em condições controladas soluções de trabalho das sondas e dos *primers* com concentrações de 10 mM e de 50 mM, respetivamente.

TABELA 20.1 Sondas e *primers* usados nas PCR em tempo real para a deteção de *F. tularensis*.

Gene/Região-alvo	Sonda/ *Primer*	Sequência (5'- 3')	Referência bibliográfica
ISFtu2	*ISFtu2-S*	AAAATCCATGCTATGACTGATGCTTTAGGTAATCCA	3
	ISFtu2-F	TTGGTAGATCAGTTGGTGGGATAAC	3
	ISFtu2-R	TGAGTTTTACCTTCTGACAACAATATTTC	3
tul4	*Tul4-S*	TTCTAAGTGCCATGATACAAGCTTCCCAATTACTAAG	3
	Tul4-F	ATTACAATGGCAGGCTCCAGA	3
	Tul4-R	TGCCCAAGTTTTATCGTTCTTCT	3
Região divergente da FPI	Tipo A-S*	AAAATTCTGC"T"CAGCAGGATTTTGATTTGGTT	6
	Tipo A-F	GAGACATCAATTAAAAGAAGCAATACCTT	6
	Tipo A-R	CCAAGAGTACTATTTCCGGTTGGT	6
	Tipo B-S*	ACCTAGTTCAACC"T"CAAGACTTTTAGTAATGGGAATGTCA	6
	Tipo B-F	CTTGTACTTTTATTTGGCTACTGAGAAACT	6
	Tipo B-R	CTTGCTTGGTTTGTAAATATAGTGGAA	6

S – sonda; F – *primer* direto; R – *primer* reverso;
*nucleótido entre aspas corresponde à posição da marcação

© LIDEL – EDIÇÕES TÉCNICAS

2. Preparar a mistura de PCR para o alvo *tul4* para um volume final, por reação, de 20 μL, contendo: 3,4 μL de água, 4 μL de $MgCl_2$ (25 mM), 2 μL da mistura de PCR (10×), 0,2 μL da sonda específica (*tul4*-S; 10 mM), e 0,2 μL de cada *primer* (*tul4*-F e *tul4*-R; 50 mM).
3. Preparar a mistura de PCR para o alvo *ISFtu2*, para um volume final, por reação, de 20 μL, contendo: 4,2 μL de água, 3,2 μL de $MgCl_2$ (25 mM), 2 μL da mistura de PCR (10×), 0,2 μL da sonda específica (*ISFtu2*-S; 10 mM), e 0,2 μL de cada *primer* (*ISFtu2*-F e *ISFtu2*-R; 50 mM).
4. Adicionar, a diferentes capilares, 10 μL das misturas de PCR preparadas nos pontos 2 e 3 anteriores.
5. Adicionar 8 μL de água estéril, livre de nucleases, a cada capilar.
6. Adicionar 2 μL de amostra de DNA em cada capilar, perfazendo assim um volume final total de 20 μL.
7. Para os controlos positivo e negativo, adicionar a cada capilar 2 μL de DNA de estirpes controlo (estirpes LVS e/ou SchuS4) e 2 μL de água estéril, respetivamente.
8. Selar os capilares utilizando a caneta e centrifugar a baixa velocidade (750–2000×g) durante 30 segundos, de modo a permitir o correto posicionamento do volume de reação no respetivo capilar.
9. Colocar os capilares no carrocel do equipamento LightCycler®, que tem a capacidade para analisar simultaneamente 32 amostras e iniciar o programa de amplificação de acordo com as instruções do fabricante. Usar o seguinte programa na análise: um passo inicial de desnaturação a 95 °C durante 10 minutos; seguido por 45 ciclos que incluem um passo de desnaturação a 95 °C durante 10 segundos e um passo de hibridação e de extensão a 60 °C durante 30 segundos; e finalmente um passo de arrefecimento a 45 °C durante 5 segundos.
10. A leitura dos resultados é realizada de acordo com as instruções do fabricante. Para os protocolos acima descritos, as sondas específicas encontram-se marcadas com o fluorocromo FAM na extremidade 5' e BBQ na extremidade 3'. A fluorescência emitida em cada ciclo da reação de amplificação é registada e apresentada graficamente em tempo real. Após análise das curvas de amplificação, indicando a presença da sequência-alvo nas amostras, a quantidade de DNA alvo nas amostras iniciais pode ser também estimada, se forem analisadas em paralelo amostras padrão de concentração conhecida.

2.2. PCR em tempo real para diferenciação de *F. tularensis* subsp. *tularensis* (Tipo A) e *F. tularensis* subsp. *holarctica* (Tipo B)

1. Preparar soluções de trabalho das sondas e dos *primers* com concentrações de 10 mM e de 50 mM, respetivamente.
2. Preparar a mistura de PCR para identificação do Tipo A, para um volume final, por reação, de 20 μL, contendo: 3,8 μL de água, 3,2 μL de $MgCl_2$ (25 mM), 2 μL da mistura de PCR (10×), 0,4 μL da sonda específica (Tipo A-S; 10 mM), e 0,3 μL de cada *primer* (Tipo A-F e Tipo A-R; 50 mM).
3. Preparar a mistura de PCR para identificação do Tipo B, para um volume final, por reação, de 20 μL, contendo: 3,8 μL de água, 3,2 μL de $MgCl_2$ (25 mM), 2 μL da mistura de PCR (10×), 0,4 μL da sonda específica (Tipo B-S; 10 mM), e 0,3 μL de cada *primer* (Tipo B-F e Tipo B-R; 50 mM).
4. Adicionar a diferentes capilares 10 μL das misturas de PCR preparadas nos pontos 2 e 3 anteriores.

© LIDEL – EDIÇÕES TÉCNICAS

5. Proceder de modo semelhante aos pontos 5 a 10 da secção 2.1. No entanto, o programa de amplificação apresenta ligeiras alterações ao usado no ponto 2.1: um passo inicial de desnaturação a 95 °C durante 10 minutos; seguido por 45 ciclos que incluem um passo de desnaturação a 95 °C durante 10 segundos e um passo de hibridação e de extensão a 65 °C durante 30 segundos; e finalmente um passo de arrefecimento a 40 °C durante 5 segundos. A sonda Tipo A encontra-se marcada com o fluorocromo FAM na extremidade 5' e BHQ na posição (nucleótido) sinalizada pelas aspas, e a sonda Tipo B encontra-se marcada com FAM na extremidade 5' e QSY-7, na posição (nucleótido) sinalizada pelas aspas (ver Tabela 20.1).

NOTAS E OBSERVAÇÕES

O protocolo de PCR em tempo real descrito neste capítulo possibilita detetar com precisão *F. tularensis* na presença de outras bactérias contaminantes, tendo-se revelado eficaz em amostras anatomopatológicas, urina, fezes e em carraças[4]. Apresenta alta especificidade, assegurada pelo uso de dois *primers* e de uma sonda específica. O protocolo de PCR em tempo real utilizando sondas de hidrólise TaqMan® tem sido referido como tendo uma sensibilidade superior à cultura, no caso de amostras complexas[4]. O alvo *tul4* é específico para *F. tularensis* sendo o tamanho do produto esperado de 91 pares de bases. O alvo *ISFtu2* garante uma sensibilidade máxima para o ensaio, apresentando um limite de deteção de um microrganismo para esta espécie, sendo o tamanho do produto esperado de 97 pares de bases. Os valores de *cycle treshold* (Ct, também designados C_q) são determinados pelo instrumento LightCycler®, para cada reação. Num estudo realizado anteriormente, quando os alvos genómicos *ISFtu2* e *tul4* foram amplificados para *F. tularensis* subsp. *tularensis*, o protocolo usando sondas de hidrólise TaqMan® demonstrou um Ct médio de 14 e 16, respetivamente, e para *F. tularensis* subsp. *holarctica* de 14 e 19, respetivamente[4].

A identificação de *F. tularensis* baseia-se na amplificação combinada de ambos os alvos, *tul4* e *ISFtu2*[4]. A nossa experiência tem demonstrado que a maior sensibilidade de amplificação do alvo *ISFtu2* permite a deteção de positivos, em amostras de tecidos de animais (com uma concentração de DNA muito baixa), na ausência de amplificação para alvo *tul4*. No entanto, a amplificação em simultâneo de alvos múltiplos aumenta a especificidade da PCR, ao reduzir a probabilidade de ocorrência de falsos -positivos, pelo que a manutenção deste ensaio combinado é sempre preferencial[4].

O protocolo usado para identificação do Tipo A apresenta um Ct médio de 17,9 e o protocolo usado na identificação do Tipo B apresenta um Ct médio de 17,1[7].

A rápida deteção de um agente infecioso como *F. tularensis*, assim como a identificação das suas subespécies com importância clínica, são críticas com vista à prevenção e/ou contenção de potenciais surtos de doença e em cenários de libertação intencional do agente. Neste sentido, os protocolos de PCR em tempo real para a deteção de *F. tularensis* aqui apresentados são imprescindíveis para a correta e atempada identificação do agente e para a adoção de medidas de controlo adequadas.

Agradecimentos

Fundação para a Ciência e a Tecnologia (FCT), entidade financiadora do projeto PTDC/SAU-ESA/104947/2008 que tem como objetivo o estudo da tularemia em Portugal; Carina Carvalho é bolseira de doutoramento FCT (SFRH/BD/79225/2011).

© LIDEL – EDIÇÕES TÉCNICAS

REFERÊNCIAS BIBLIOGRÁFICAS

1 Ahlinder J, Ohrman C, Svensson K et al. (2012). Increased Knowledge of *Francisella* genus diversity highlights the benefits of optimized DNA-based assays. *BMC Microbiol.* 12:220.

2 Euler M, Wang Y, Otto P et al. (2012). Recombinase polymerase amplification assay for rapid detection of *Francisella tularensis. J Clin Microbiol.* 50:2234–2238.

3 Carvalho CL, Lopes de Carvalho I, Zé-Zé L, Núncio MS, Duarte EL (2014). Tularaemia: A challenging zoonosis. *Comp Immunol Microbiol Infect Dis.* 37:85–96.

4 Vogler AJ, Birdsell D, Wagner DM, Keim P (2009). An optimized, multiplexed multi-*locus* variable-number tandem repeat analysis system for genotyping *Francisella tularensis. Lett Appl Microbiol.* 48:140–144.

5 Versage JL, Severin DD, Chu MC, Petersen JM (2003). Development of a multitarget real-time TaqMan PCR assay for enhanced detection of *Francisella tularensis* in complex specimens. *J Clin Microbiol.* 41:5492–5499.

6 Johansson A, Farlow J, Larsson P et al. (2004). Worldwide genetic relationships among *Francisella tularensis* isolates determined by multiple-*locus* variable-number tandem repeat analysis. *J Bacteriol.* 186:5808–5818.

7 Kugeler KJ, Pappert R, Zhou Y, Petersen JM (2006). Real-time PCR for *Francisella tularensis* types A and B. *Emerg Infect Dis.* 12:1799–1801.

8 Chistensen DR, Hartman LJ, Loveless BM et al. (2006). Detection of biological threat agents by real-time PCR: comparison of assay performance on the R.A.P.I.D., the LightCycler, and the Smart Cycler platforms. *Clin Chem.* 52:141–145.

9 Larsson P, Svensson K, Karlsson L et al. (2007). Canonical insertion-deletion markers for rapid DNA typing of *Francisella tularensis. Emerg Infect Dis.* 13:1725–1732.

10 Michelet L, Bonnet S, Madani N, Moutailler S (2013). Discriminating *Francisella tularensis* and *Francisella*-like endosymbionts in *Dermacentor reticulatus* ticks: evaluation of current molecular techniques. *Vet Microbiol.* 163:399–403.

11 Nano FE, Zhang Na, Cowley SC et al. (2004). A *Francisella tularensis* pathogenicity island required for intramacrophage growth. *J Bacteriol.* 186:6430–6436.

© LIDEL – EDIÇÕES TÉCNICAS

Study 10

Is *in vivo* passage useful in *F. tularensis* isolation in the presence of low bacteria loads?

Isolation attempt of *Francisella tularensis holarctica* from *Oryctolagus cuniculus* and *Lepus granatensis* tissues after an *in vivo* passage in CD1 mice

Carina L. Carvalho[1], Líbia Zé-Zé[2], Elsa L. Duarte[1,3] Isabel Lopes de Carvalho[2,4]

[1]Instituto de Ciências Agrárias e Ambientais Mediterrânicas (ICAAM), Universidade de Évora, Évora, Portugal; [2]Centro de Estudos de Vectores e Doenças Infecciosas (CEVDI), Instituto Nacional de Saúde Doutor Ricardo Jorge (INSARJ), Águas de Moura, Portugal; [3]Departamento de Medicina Veterinária, Universidade de Évora, Évora, Portugal;
[4]Unidade de Resposta de Emergência e Biopreparação (UREB), Instituto Nacional de Saúde Doutor Ricardo Jorge (INSARJ), Lisboa, Portugal

Manuscript in preparation

1. Abstract

The zoonotic bacterium *Francisella tularensis* has been detected in Portugal in several tick species and recently also in wild lagomorphs. Sequencing analysis allowed identifying *F. tularensis* subsp. *holarctica* circulating in the country, although further characterization is still needed. Since the first detection of this bacterium in Portugal, several *F. tularensis* isolation attempts have been made by the authors, resorting to standard protocols referred in the literature. However, all isolation attempts failed. In this work, we used an alternative protocol to propagate *F. tularensis "in vitro"* in suitable cysteine enriched medium, which includes a previous *"in vivo"* passage in mice (CD1). This method was developed by Gyuranecz (2011) and have been applied with success for the *F. tularensis* isolation at the Institute for Veterinary Medical Research (Center for Agricultural Research, Hungarian Academy of Sciences), a OIE *F. tularensis* reference laboratory.

2. Keywords: *Francisella tularensis, F. tularensis* subsp. *holarctica*, *in vitro* propagation, *in vivo* passage, isolation attempt, cultivation

3. Body of manuscript

In Portugal the zoonotic bacterium *Francisella tularensis* was first detected in 2007 by molecular methods in the blood of an asymptomatic man and in a *Dermacentor reticulatus* tick (Lopes de Carvalho et al., 2007). Since then, the Centre for Vectors and Infectious Diseases Research (CEVDI), National Health Institute Doutor Ricardo Jorge (INSARJ), the Portuguese reference laboratory on *F. tularensis*, has reinforced surveillance on this pathogen. Recently, alongside with ticks from genera *Dermacentor* and *Ixodes*, the bacterium was also identified in the country in *Hyalomma lusitanicum* and *Rhipicephalus sanguineus* tick species as well as in wild lagomorphs (Lopes de Carvalho et al., 2016). Sequencing analysis disclosed the circulation of *F. tularensis* subsp. *holarctica* in Portugal.

F. tularensis is a zoonotic bacterium responsible for tularaemia (Petersen et al., 2009). Although it was isolated in 1912 in California, a growing research interest on this pathogen was registered last years due to its increasing emergence in new hosts and geographic locations, but also for its potential use in bioterrorism (reviewed in (Carvalho et al., 2014)). *F. tularensis* is considered one of the most virulent microorganisms presently known, being classified as a Category A biowarfare agent (Dennis et al., 2001). In this context a rapid and unequivocal laboratorial diagnosis is often required, especially when *Francisella*-like endosymbionts with significant homology to *F. tularensis* have been described (Scoles, 2004)(Escudero et al., 2008)(Baldridge et al., 2009)(de Carvalho et al.,

2011)(Ivanov et al., 2011)(Dergousoff and Chilton, 2012)(Kreizinger et al., 2013). Also, *F. tularensis* is formally divided into three subspecies with different pathogenicity and geographic distribution, namely *F. tularensis* subsp. *tularensis*, *F. tularensis* subsp*. holarctica and F. tularensis* subsp. *mediasiatica*. Considering the subspecies with pathogenicity to humans, although *F. tularensis* subsp. *tularensis* is considered the most virulent (Type A), the less virulent *F. tularensis* subsp. *holarctica*, (Type B) has a wider distribution (reviewed in (Carvalho et al., 2014)) making important to discriminate between them (Versage et al., 2003)(Escudero et al., 2008). Hence, a Taqman probe real time multiplex assay, with high specificity and sensitivity for *F. tularensis* identification was developed (Versage et al., 2003) as well as a real-time assay for subspecies discrimination (Kugeler et al., 2006). Still, culture is the gold standard for *F. tularensis* laboratorial identification (Tärnvik, 2007). Furthermore, although molecular techniques such as metagenomic sequencing using Next Generation Sequencing can provide important information on a certain microrganism in the absence of culture (Stewart et al., 2012)(Albanese et al., 2015)(Antwerpen et al., 2015), only its isolation in pure culture allows a comprehensive characterization of the physiological properties and a full assessment of the virulence potential (Vartoukian et al., 2010). *F. tularensis* is, however, a fastidious microorganism having specific growth requirements including a cysteine supplemented growth medium. Optimal growth conditions occur at 37°C and pH 6.9 (Splettstoesser et al., 2005)(Foley and Nieto, 2010) and cysteine-enriched media, such as enriched chocolate agar (CA) or 9% cysteine heart agar with blood medium (CHAB) must be used (Splettstoesser et al., 2005) (Tärnvik, 2007). CHAB medium enables the presumptive identification of *F. tularensis* by characteristic growth at 24-48h of round and smooth green opalescent shiny colonies, 2-4 mm in diameter (Splettstoesser et al., 2005)(Tärnvik, 2007). Antibiotic supplementation can optimise growth and inhibit contaminants (Splettstoesser et al., 2005). *F. tularensis* isolation must be carried out in biosecurity level 3 facilities (BSL-3) (Tärnvik, 2007).

Since *F. tularensis* was first detected in Portugal, several isolation attempts by the authors using field samples, mostly ticks but recently also organs tissues from wild lagomorphs, have failed hampering the full characterization of circulating strains. Although several protocols referred in the literature have been tried, no isolation attempt was successful, probably due to the low bacteria present in the samples.

In this work, we aimed to propagate *F. tularensis "in vitro"* in cysteine enriched medium (PolyViteX^{TM}, Biomérieux®), after an *in vivo* passage in mice following the protocol developed by Gyuranecz (2011) and applied with success in the *F. tularensis* isolation in the Institute for

Veterinary Medical Research (Center for Agricultural Research, Hungarian Academy of Sciences), recently accredited by the OIE *as F. tularensis* reference laboratory.

Liver samples from three wild lagomorphs specimens collected in a hunting station in the Alto Alentejo region were selected for this study. Two of the liver samples originated from *Lepus granatensis* and one from *Oryctolagus cuniculus* species. All samples tested positive for *F. tularensis* in the real-time multitarget PCR described by Versage et al. (2003) and were identified as *F. tularensis* subsp. *holarctica* by the real time assay developed Kugeler et al. (2006). Samples Cq values were all above 34.11, hence close to the cut-off. The protocol described below was carried out in BSL3 facilities, under supervision of an animal experimentation certified expert to assure animal welfare.

Homogenates of liver samples (0.25 grams (gr)) were prepared in physiological saline solution (NaCl, 9% w/v) (0.5 mL). The homogenate was vortexed and transferred to a 2 mL syringe with a 20 gauge needle. Three CD1 mice weighting approximately 20 gr each were identified in accordance with the sample. The totality of each inoculum was administrated subcutaneously to the respective animal. Mice were observed three times per day during 10 consecutive days for the presence of any abnormal clinical sign. In the absence of any symptomatology, mice were sacrificed at day 10 post-inoculation in a CO_2 chamber, and organs (heart, lungs, liver, spleen and kidney) and blood samples were collected. Each organ was then macerated and used to inoculate chocolate agar PolyViteX™ (BioMerieux®,) comercial plates. Plates were observed at 24, 48 and 72 hours post-inoculation. Developing colonies were identified by morphological characteristics; none however presented the characteristic growth of *F. tularensis* referred above. Nevertheless, each colony was transferred to an eppendof containing 180 µL of ATL and 20 µL of proteinase K (Qiagen, Germany). Spin column protocols were used for genomic DNA extraction (Qiagen, Germany), following the manufactures' recommendations. DNA samples were screened for *F. tularensis* using the TaqMan real-time PCR targeting *tul*4 gene and the insertion element *ISFTu*2 described by Versage et al. (2003). All samples tested negative for the presence of *F. tularensis*.

Later, another set of four samples, including spleen (n=2) lung (n=1) and liver (n=1), all from *O. cuniculus*, that tested positive for *F. tularensis* in the real time multitarget PCR described by above, were also investigated using the same protocol. Again, no isolation of *F. tularensis* was obtained.

Several factors were identified as possible causes of the unsuccessful isolation of *F. tularensis*.

Firstly, although all samples used in this work tested positive for *F. tularensis* by a specific and sensitive real-time PCR, all the Cqs obtained were above 34.11, implying that the amount of DNA

present in the tissues was very low and not necessarily representing viable *F. tularensis* cells. The absence of viable bacteria would totally compromise the isolation. Also, in the protocol followed by Gyuranecz and collaborators, *F. tularensis* samples present in macroscopic lesions in parenchymal organs are collected for homogenates preparation. According to the experience of Gyuranecz and collaborators (*personal communication*) the probability of *F. tularensis* successful isolation by this methodology is greatly compromised in the absence of macroscopic lesions in target organs, despite positiveness in molecular tests. This was the case of this work, as no macroscopic lesions could be observed in the organs. Furthermore, Gyuranecz (2011) recommend the used of 1 gr of sample tissue, containing visible lesions, macerated in 2 mL of physiological saline solution (NaCl 9%) and the subcutaneously administration of 1 mL of inoculum per mouse. In this work, homogenates preparation used on average quarter of the amount recommended by Gyuranecz (2011) due to sample limitation, which certainly reduced the number of viable bacteria in the inoculum, their replication in the hosts' tissues and subsequent isolation.

No serial *in vivo* passages were carried out in any of the experiments described in this work. Hence wheter this approach would have increased bacteria replication in the hosts' tissues leading to successful of isolation is yet to be clarified.

While *F. tularensis* isolation remains to be achieved, the full characterization of the strains circulating in Portugal greatly relies in new generation sequencing techniques.

4. Acknowledgments

The authors thank to Doctor Maria João Alves for her orientation during the animal experimentation and Ana Mar for her technical assistance. This study was partially funded by a grant from the Fundação para a Ciência e Tecnologia (FCT) SFRH/79225/2011.

5. References

Carvalho, C.L., Lopes de Carvalho, I., Zé-Zé, L., Núncio, M.S., Duarte, E.L., 2014. Tularaemia: A challenging zoonosis. Comp. Immunol. Microbiol. Infect. Dis. 37, 85–96. doi:10.1016/j.cimid.2014.01.002

Dennis, D.T., Inglesby, T. V, Henderson, D.A., Bartlett, J.G., Ascher, M.S., Eitzen, E., Fine, A.D., Friedlander, A.M., Hauer, J., Layton, M., Lillibridge, S.R., McDade, J.E., Osterholm, M.T., O'Toole, T., Parker, G., Perl, T.M., Russell, P.K., Tonat, K., Working Group on Civilian Biodefense, 2001. Tularemia as a biological weapon: medical and public health

management. JAMA 285, 2763–73.

Foley, J.E., Nieto, N.C., 2010. Tularemia. Vet. Microbiol. 140, 332–338. doi:10.1016/j.vetmic.2009.07.017

Lopes de Carvalho, I., Escudero, R., García-Amil, C., Falcão, H., Anda, P., Núncio, M.S., 2007. Francisella tularensis, Portugal. Emerg. Infect. Dis. 13, 666–667. doi:10.3201/eid1304.060714

Lopes de Carvalho, I., Toledo, A., Carvalho, C.L., Barandika, J.F., Respicio-Kingry, L.B., Garcia-Amil, C., García-Pérez, A.L., Olmeda, A.S., Zé-Zé, L., Petersen, J.M., Anda, P., Núncio, M.S., Escudero, R., 2016. Francisella species in ticks and animals, Iberian Peninsula. Ticks Tick. Borne. Dis. 7, 159–65. doi:10.1016/j.ttbdis.2015.10.009

Gyuranecz M. 2011. Epizootic investigations of tularemia and the comparative characterization of *Francisella tularensis* strains. Ph.D. dissertation

Petersen, J.M., Mead, P.S., Schriefer, M.E., 2009. Francisella tularensis: an arthropod-borne pathogen. Vet. Res. 40, 7. doi:10.1051/vetres:2008045

Splettstoesser, W.D., Tomaso, H., Al Dahouk, S., Neubauer, H., Schuff-Werner, P., 2005. Diagnostic procedures in tularaemia with special focus on molecular and immunological techniques. J. Vet. Med. B. Infect. Dis. Vet. Public Health 52, 249–61. doi:10.1111/j.1439-0450.2005.00863.x

Tärnvik, A., 2007. WHO Guidelines on Tularaemia. Geneva: WHO/CDS/EPR/2007.7

Chapter VII

Epidemiology of *Francisella tularensis*

1. Update on the epidemiology of *Francisella tularensis*

In addition to the information gathered on the *F. tularensis* epidemiology that is presented in General Introduction, some important pieces of information will be added in this introduction.

Despite all ages are equally susceptible to tularaemia, young children and young adults are more likely to participate in activities that make them more susceptible to exposure. Risk factors associated with tularaemia involve certain hobbies and/or occupations including hunting, gardening, landscaping and wildlife work (Anand et al., 2017).

1.1. Mammalian reservoir hosts

The results of the surveillance of tularaemia in wildlife in France (between the 2002-2003 and 2012-2013 hunting seasons) investigating the spatial and temporal pattern of the disease were recently published (Moinet et al., 2016). These results revealed that the main species affected by tularaemia in France in the period considered was the European Brown Hare (*Lepus europaeus*). However, wild rabbits (*Oryctolagus cuniculus*, n=4), roe deers (*Capreolus capreolus*, n=2) and wild boars (*Sus scrofa*, n=1)) were also found positive (Moinet et al., 2016). The detection of *F. tularensis* subsp. *holarctica* in wild rabbits is in accordance with the results recently published by our team reporting for the first time the detection of this pathogen in *Oryctolagus cuniculus* (Lopes de Carvalho et al., 2016). The results of this surveillance study also confirmed the usefulness of the brown hare as a sentinel of environmental risk regarding tularaemia (Moinet et al., 2016).

In their recent phylogeographic study, Dwibedi et al. (2016) suggested simultaneously the long-term persistence of *F. tularensis* in some regions as well as long range dispersal of this pathogen as an important feature of tularaemia ecology. Their results supported a model of *F. tularensis* biology involving outbreaks restricted to specific stationary ecosystems and landscapes, indicating that the pathogen is dependent on particular local ecological conditions where it has the ability to persist and cause repeated outbreaks (Pavlovsky, 1966)(Goethert and Telford, 2009)(Svensson et al., 2009). In addition, the Dwibedi et al. (2016) results also suggested very long-distance and rapid movements of *F. tularensis*, although the mechanisms underlying this long-range dispersal are unknown. It is proposed that bacteria may move rapidly through different settings by infected domestic or wild animals, or windborne (Burrows et al., 2009). Even if geographical migration of the European hare (*L. europaeus*) is restricted, conservation actions and translocations of animals may transfer infected hares from tularaemia-endemic areas to previously tularaemia-free areas (Ferretti

et al., 2010)(Petersen and Schriefer, 2005). This has been suggested for the emergence of the disease in Spain in the 1990s (Petersen and Schriefer, 2005). Migratory birds have also been proposed to be involved in the long distance spread of *F. tularensis* was also (Lopes de Carvalho et al., 2012). Another possible mechanism for the long-distance transmission of this pathogen is windborne dispersal (Dennis et al., 2001). Considering *F. tularensis* propensity to be part of aerosols and its environmental resistance, long-distance dispersal of this pathogen may occur (Nguyen et al., 2006)(Smith et al., 2013).

1.2. Arthropod vectors

The transmission of tularaemia to humans by arthropod vectors is especially evident in endemic areas, such as Turkey, Sweden, and certain regions of the United States (Mörner, 1992)(Feldman et al., 2001)(Sjöstedt, 2007).

Ticks are the most extensively studied known vectors of tularaemia (Kenney et al., 2017) and monitoring them regarding *F. tularensis* cell numbers of per tick may be a valuable tool in the surveillance of the disease, as pointed out recently by Hubálek and Rudolf (2017), that surveyed *D. reticulatus* ticks and quantified *F. tularensis* cells per tick. Even in countries were *F. tularensis* has not been detected for more than two decades in ticks, the need for this surveillance is evidenced by a recent study carried out in Japan. Despite *F. tularensis* had not been detected in ticks in Japan since 1991, the pathogen (*F. tularensis* Type B) was recently detected by molecular methods in *Ixodes ovatus*, *I. persulcatus*, *I. monospinosus* and *Haemaphysalis flava* specimens, suggesting that these act as vectors for *F. tularensis* infection in that country (Suzuki et al., 2016).

In addition to ticks, mosquitoes are vectors of *F. tularensis*, particularly in the Scandinavian countries, where they are considered the main source of tularaemia (Christenson, 1984)(Desvars et al., 2015)(Payne et al., 2005)(Tärnvik et al., 1996). Prior research demonstrated the presence of *Francisella* sp. DNA in infected mosquitoes, but has failed to prove definitive transmission of tularaemia from mosquito to mammalian hosts. According to Kaushal et al. (2016), mosquito antimicrobial peptides act against *Francisella* sp. by disrupting the bacterial cellular membrane and may play a role in mosquitos' inability in establishing an effective natural transmission. Besides being mechanical vectors, mosquitoes are important vectors of *F. tularensis* and have been related to significant tularaemia outbreaks (Christenson, 1984) and more than 10 different mosquito species have been identified to harbour and potentially transmit *F. tularensis* (Thelaus et al., 2014). Recently, Kenney et al. (2017) aiming at evaluating the factors contributing to the capacity of

mosquitoes to maintain and spread tularaemia in nature, proposed that the flower nectar could act as a temporary source of *F. tularensis* increasing the ability of mosquitoes to spread this bacterium. According to these researchers, the flower nectar (including the one from *Lappula deflexa* and *Dianthus arenarius,* among others) may serve as a temporary source of *F. tularensis*, as the bacterium was proven to survive in flower nectar over a period of time consistent with that of a typical flower bloom. This would increase the ability of mosquitoes to spread this bacterium in nature and contribute to the amplification of outbreaks. Kenney et al. (2017) hypothesized that flower nectar may allow vector-vector transmission of *F. tularensis*, as female mosquitoes feeding on infected mammalian hosts and subsequently on flower nectar could deposit the pathogen and mosquitoes subsequently feeding on nectar could potentially become colonized by *F. tularensis*.

Study 11

In which mammalian species and vectors do *F. tularensis* circulates in the Iberian Peninsula?

Francisella species in ticks and animals, Iberian Peninsula

Isabel Lopes de Carvalho[1], Alvaro Toledo[2,3,4], Carina L. Carvalho [5,6], Jesse F. Barandika[7], Laurel B. Respicio-Kingry[8], Cristina Garcia-Amil C[2], Ana L. García-Pérez[7], Angeles S. Olmeda[3], Líbia Zé-Zé[9], Jeanine M. Petersen[8], Pedro Anda[2], Maria S. Núncio[1,6], Raquel Escudero[2]

[1]Instituto Nacional de Saúde Dr. Ricardo Jorge, Lisbon, Portugal; [2]Instituto de Salud Carlos III, Majadahonda, Madrid, Spain; [3]Universidad Complutense de Madrid, Madrid, Spain; [4]Current affiliate: Stony Brook University, Stony Brook, New York, USA; [5]Instituto de Ciências Agrárias e Ambientais Mediterrânicas (ICAAM), Universidade de Évora, Portugal; [6]Instituto Nacional de Saúde Doutor Ricardo Jorge (INSARJ), Águas de Moura, Portugal; [7]NEIKER - Instituto Vasco de Investigación y Desarrollo Agrario, Derio, Biscay, Spain; [8]Centers for Disease Control and Prevention, Fort Collins, Colorado, United States of America;

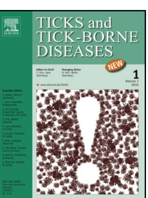
Ticks Tick Borne Diseases. 7(1):159-65 doi: 10.1016/j.ttbdis.2015.10.009

Ticks and Tick-borne Diseases 7 (2016) 159–165

Contents lists available at ScienceDirect

Ticks and Tick-borne Diseases

journal homepage: www.elsevier.com/locate/ttbdis



Original article

Francisella species in ticks and animals, Iberian Peninsula

I. Lopes de Carvalho[a,∗,1], A. Toledo[b,c,1,2], C.L. Carvalho[d,e], J.F. Barandika[f], L.B. Respicio-Kingry[g], C. Garcia-Amil[b], A.L. García-Pérez[f], A.S. Olmeda[c], L. Zé-Zé[d], J.M. Petersen[g], P. Anda[b], M.S. Núncio[a,d], R. Escudero[b]

[a] Instituto Nacional de Saúde Dr. Ricardo Jorge, Lisbon, Portugal
[b] Instituto de Salud Carlos III, Majadahonda, Madrid, Spain
[c] Universidad Complutense de Madrid, Madrid, Spain
[d] Instituto Nacional de Saúde Dr. Ricardo Jorge, Águas de Moura, Portugal
[e] University of Évora, Évora, Portugal
[f] NEIKER – Instituto Vasco de Investigación y Desarrollo Agrario, Derio, Biscay, Spain
[g] Centers for Disease Control and Prevention, Fort Collins, CO, USA

ARTICLE INFO

Article history:
Received 27 April 2015
Received in revised form 24 August 2015
Accepted 14 October 2015
Available online 19 October 2015

Keywords:
Francisella tularensis subsp. *holarctica*
Francisella-like endosymbionts
Francisella hispaniensis
Ticks
Lagomorphs
Mammals
Spain
Portugal

ABSTRACT

The presence of *Francisella* species in 2134 ticks, 93 lagomorphs and 280 small mammals from the Iberian Peninsula was studied. Overall, 19 ticks and 6 lagomorphs were positive for *Francisella tularensis* subsp. *holarctica*, suggesting, as described for other regions, that lagomorphs may have an important role in the maintenance of *F. tularensis* in nature. Of the 6 positive lagomorphs, 4 were identified as the European rabbit, *Oryctogalus cuniculus*. Additionally, 353 ticks and 3 small mammals were PCR positive for *Francisella*-like endosymbionts (FLEs) and one small mammal was also positive for *Francisella hispaniensis*-like DNA sequences. Among FLE positive specimens, a variety of sequence types were detected: ticks were associated with 5 *lpnA* sequence types, with only one type identified per tick, in contrast to 2 *lpnA* sequence types detected in a single wood mouse (*Apodemus sylvaticus*). To our knowledge, this is the first report of FLEs in free-living small mammals as well as the first detection of *F. hispaniensis*-like sequences in a natural setting.

© 2015 Elsevier GmbH. All rights reserved.

1. Introduction

Francisellaceae comprise a bacterial family widespread in nature with several new species identified in recent years. The most widely recognized species within the *Francisella* genus is *Francisella tularensis*, the causative agent of tularemia, a disease that can be lethal to humans and to a wide variety of animal species (Dennis et al., 2001; Sjöstedt, 2005). Tularemia can be transmitted by the inhalation of contaminated aerosols, direct contact with infected sources, ingestion of contaminated food or water, and by the bite of infected arthropods such as ticks and mosquitoes that can act as vectors of the microorganism (Ellis et al., 2002).

Francisella hispaniensis is a more recently described member of the genus, initially described after isolation from a patient in Spain with severe illness (Huber et al., 2010). Currently, its distribution in nature is unknown.

Recent whole genome sequencing of the tick endosymbiont, *Wolbachia persica* verified the taxonomic position of *Francisella*-like endosymbionts (FLEs) within the *Francisella* genus (Niebylski et al., 1997; Sjödin et al., 2012). FLEs have been described to date in different tick species including *Dermacentor*, *Amblyomma*, *Ornithodoros*, *Ixodes* and *Hyalomma* and are thought to be restricted to this host (Niebylski et al., 1997; Sun et al., 2000; Scoles, 2004; de Carvalho et al., 2011; Machado-Ferreira et al., 2009; Kreizinger et al., 2013; Szigeti et al., 2014; Wójcik-Fatla et al., 2015). In 1997, Niebylski et al. demonstrated that wood ticks harbored a *Francisella*-like organism in ovarial tissues, termed the *Dermacentor andersoni* symbiont (DAS), which failed to be horizontally transmitted when infected ticks were fed on guinea pigs. It was stably maintained both transovarially and transtadially in these ticks suggesting that the DAS is a tick endosymbiont whose biology contrasts with *F. tularensis* (Niebylski et al., 1997). Consistent with

∗ Corresponding author at: Instituto Nacional de Saúde Doutor Ricardo Jorge I.P., Departamento de Doenças Infecciosas, Unidade de Resposta a Emergências e Biopreparação, Av. Padre Cruz, 1649-016 Lisboa, Portugal.
E-mail address: isabel.carvalho@insa.min-saude.pt (I. Lopes de Carvalho).
[1] These authors contributed equally to this article.
[2] Current address: Stony Brook University, Stony Brook, New York, USA.

http://dx.doi.org/10.1016/j.ttbdis.2015.10.009
1877-959X/© 2015 Elsevier GmbH. All rights reserved.

the designation of FLEs as tick symbionts, the genome size of *W. persica* was shown to be significantly reduced (about 80–85%) as compared to other *Francisella* genomes.

Arguably, ticks are the most important arthropods in the ecology of tularemia. In Europe, *Dermacentor marginatus*, *Dermacentor reticulatus*, *Ixodes ricinus* and *Haemaphysalis concinna* ticks have been reported to harbor *F. tularensis* (Toledo et al., 2009; Franke et al., 2010; Gyuranecz et al., 2011). In Portugal, *F. tularensis* subsp. *holarctica* was first reported in *D. reticulatus* and in humans in 2007 (de Carvalho et al., 2007) whereas FLEs were first detected in *D. reticulatus* in 2010 (de Carvalho et al., 2011). In Spain, sporadic human cases of tularemia associated with tick bite have been reported (Teijo-Núñez et al., 2006), since the first reported outbreak due to *F. tularensis* subsp. *holarctica* in 1997 (Eiros Bouza and Rodríguez-Torres, 1998; Anda et al., 2001). FLEs have been regularly identified in a variety of tick species in Spain since their first detection in 2008 (Escudero et al., 2008).

Here we report the presence of *F. tularensis* subsp. *holarctica* in lagomorphs, small mammals and different tick species from the Iberian Peninsula. We also report for the first time DNA detection of *F. hispaniensis*-like organisms and *Francisella*-like endosymbionts in small mammals.

2. Material and methods

2.1. Tick and animal collection and identification

Ticks were collected from vegetation in different areas in Portugal (two northern districts, Bragança and Vila Real, and one southern, Évora) and Spain (northern Basque Country and the centrally located Madrid region). Sampling sites were selected to account for geographic, climatic, botanic and land-use differences. Sampling sites in both areas have been fully described previously (Barandika et al., 2011). Domestic herbivores and wildlife species were present in all the zones. According to the Köppen-Geiger climate classification, central Spain and Portugal have Mediterranean climates (Csa/Csb) whereas northern Spain has an oceanic climate (Cfb) (Peel et al., 2007). Central Spain and Portugal areis comprised of forests, woodlands, and scrub, with the predominant animals being free-living wildlife species.

Briefly, a 2 × 1.6-m white blanket was dragged for 30 min, and ticks attached to the blanket were collected every 5 min. Also, ticks feeding on wild and domestic animals were manually removed and placed into an empty tube and stored at −80 °C until further use. All specimens were identified to the species level by using appropriate taxonomic keys (Manilla, 1998). Rodents from the same areas were trapped using Sherman (H.B. Sherman Traps, Tallahasee, FL) and INRA traps (BTS Mechanique, Besançon, France). Also, during the annual hunting season in 2011 and 2012 in Evora (South of Portugal), tissues (liver and spleen) were collected from European brown hares (*Lepus europaeus*) and European rabbits (*Oryctolagus cuniculus*) at a hunter check station. The animals were identified (MacDonald and Barrett, 1993), necropsied and tissues were individually stored at −20 °C. All tick and animal samples were collected in different regions and at different times, and share no direct correlation.

2.2. DNA extraction, polymerase chain reactions and sequencing

Adult ticks were classified by species and processed individually. Ticks were washed sequentially with distilled water, 10% hydrogen peroxide and 70% ethanol, for 5 min each, and DNA was extracted from 200 μL of PBS cell suspension by using the DNeasy tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. The same DNA extraction procedure was also used for animal tissues. Extracted DNA was stored at −20 °C until analyzed by PCR.

Samples from Portugal were tested using a real-time multitarget TaqMan PCR, using *tul4* and IS*Ftu2* assays (Versage et al., 2003) with positives further tested using real-time TaqMan PCR assays which differentiate between *F. tularensis* subsp. *tularensis* (type A) and *F. tularensis* subsp. *holarctica* (type B) (Kugeler et al., 2006). For testing of samples from Spain, a phylogenetically informative region of *lpnA* (231 bp) was amplified by conventional PCR as previous described (Escudero et al., 2008). For additional characterization, a portion of the succinate dehydrogenase A (*sdhA*) gene and VNTR Ft-M19 were also amplified (Byström et al., 2005; Barns et al., 2005; Berrada and Telford, 2010). A negative PCR control as well as a negative control for DNA extraction was included in each group of samples processed. For real-time PCR using *tul4*, IS*Ftu2*, type A and type B assays, a type A positive control was used, as type A strains are restricted to North America All negative controls demonstrated no PCR amplification.

Amplicons obtained by *lpnA*, *sdhA* and Ft-M19 PCR were purified using the Jetquick Purification PCR Product Spin kit (Genomed Inc., Lohne, Germany) and sequenced with the ABI BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Inc., Foster City, CA, USA). Samples that showed mixed signals in the sequence chromatograms were cloned in pGEM-T-easy vectors (Promega Biotech Ibérica, SL, Madrid, Spain), following the manufacturer's instructions, and eight clones of each were sequenced.

The *lpnA* and *sdhA* sequences for *F. tularensis* subsp. *holarctica* (PoTiF2, PoTiF3, PoAnF2), *F. hispaniensis*-like (AR1MM2), *Francisella*-like endosymbiont (PoTiEF2, PoTiEF3, PoAnEF1, AR1MM1, AR1MM2, GU1MM1, ST1, ST2, ST4) were assigned the following GenBank accessions numbers respectively: KJ477079, KJ477084, KJ477081, KJ734992, KJ477082, KJ77083, EU315914, EU315916, EU315913, EU315915, EU315911, EU315912, KJ689454.

2.3. Phylogenetic analysis

DNA sequencing was repeated at least two times to verify the nucleotide position. Sequences were assembled by combining the sequences generated by each primer using the BioEdit software (Ibis Biosciences, Inc., Carlsbad, CA, USA). All alignments were made using ClustalW program (Thompson et al., 1994) and manually inspected for misalignments. FLE sequence types were determined after sequence alignment via identification of polymorphic positions. A neighbor-joining tree of DNA sequence alignment was built in MEGA version 6 (Tamura et al., 2013). Distance matrices were calculated using the Kimura two-parameter model to correct for multiple substitutions. Bootstrap analysis was obtained with 1000 replicates.

3. Results

3.1. Detection of Francisella in ticks and animals

Overall, from both Spain and Portugal, 2134 ticks were tested, with 19 (0.9%) positive for *F. tularensis* subsp. *holarctica* and 353 (16.5%) positive for FLEs. From Spain, 13 different tick species from 5 genera were collected (1897 specimens: 1454 from vegetation and 443 from animals) (Tables 1 and 2). Of these, 1 questing tick (1 *Hyalomma lusitanicum*; 0.06%) and 3 ticks collected from animals (2 *D. marginatus* and 1 *Rhipicephalus sanguineus*; 0.6%) were positive for *F. tularensis* subsp. *holarctica* (Tables 1 and 2). In contrast, a total of 348 ticks representing 6 species were positive for FLEs, with an overall positivity rate of 18.3% (Tables 1 and 2). The two tick species, *H. lusitanicum* and *D. reticulatus* accounted for the vast majority (93%) of FLE positives. From Portugal, a total of 237

Table 1
Francisella tularensis subsp. *holarctica* (Fth) and *Francisella*-like endosymbiont (FLE) positives in questing ticks analyzed.

	Madrid				Basque Country				Portugal			
	N	Positive (%)[a]	Fth[b]	FLE (*lpnA* sequence type)	*N*	Positive (%)[a]	Fth[b]	FLE (*lpnA* sequence type)	*N*	Positive (%)[c]	Fth[d]	FLE (*lpnA* sequence type)
Hyalomma lusitanicum	701	173 (24.7)	1 (0.14)	172 (ST1)	0				0			
Rhipicephalus pusillus	47	0			0				0			
Dermacentor reticulatus	0	0			97	81 (83.5)		81 (ST4)	42	5 (11.9)	1 (2.4)	4 (ST4)
Rhipicephalus bursa	16	0			50	0			0			
Ixodes ricinus	8	0			288	0			12	3 (25)	3 (25)	
Haemaphysalis punctata	1	0			109	0			0			
Haemaphysalis hispanica	1	0			0				0			
Haemaphysalis inermis	0				95	0			0			
Haemaphysalis concinna	0				41	0			0			
Dermacentor marginatus	0				0				82	2 (2.4)	2 (2.4)	
Ixodes hexagonus	0				0				38	1 (2.6)		1 (ST4)
Hyalomma marginatum	0				0				1	0		
Rhiphicephalus sanguineus	0				0				35	9 (25.7)	9 (25.7)	
Total	774	173 (22.3)	1 (0.12)	172 (22.2)	680	81 (11.9)	0	81 (100)	210	20 (9.5)	15 (7.1)	5 (2.3)

[a] Ticks were considered *Francisella* PCR positive by testing of the lpnA gene.
[b] Ticks were considered *F. tularensis* subsp. *holarctica* positive by sequencing the lpnA gene.
[c] Ticks were considered *Francisella* PCR positive by testing of the *lpnA* gene or by real-time PCR testing of the *tul4* and ISFtu2 targets.
[d] Ticks were considered *F. tularensis* subsp. *holarctica* positive by testing with the real-time Type B subtyping assay, *lpnA* and/or FT-M19.

ticks encompassing 7 tick species of four different genera were collected (237 ticks: 210 from vegetation, and 27 from animals) (Tables 1 and 2). Of these, 15 questing ticks (2 *D. marginatus*, 1 *D. reticulatus*, 3 *I. ricinus* and 9 *R. sanguineus*; 6.3%) tested positive for *F. tularensis* subsp. *holarctica.* Additionally, 5 questing ticks representing 2 species (4 *D. reticulatus* and 1 *I. hexagonus*) were positive for FLEs, with a positivity rate of 2.4%.

Of the 373 animals (10 different species) tested, 6 tested PCR positive for *F. tularensis* subsp. *holarctica*. All six positives were among the 93 lagomorphs (no small mammals; 14 *L. europaeus* and 79 *O. cuniculus*) tested from Portugal (Table 3). Of the 6 positive lagomorphs, 4 were *O. cuniculus* and 2 were *L. europaeus*. One additional lagomorph tested positive for *Francisella* sp., but it was not possible to identify to the species level via sequencing of the *sdhA* and *lpnA* genes. From Spain, a total of 280 small mammals were tested with none positive for *F. tularensis*. Interestingly, three *A. sylvaticus* (1.1%) tested positive for FLEs in liver, kidney and spleen, respectively. Moreover, in one of these animals, a *lpnA* DNA sequence most similar (98.5% identity) to *F. hispaniensis* was also detected (Table 3, Fig. 1). Attempts to sequence an additional gene were unsuccessful.

3.2. Sequencing and phylogenetic analysis

Among the FLE positive samples, sequence analysis of the *lpnA* gene identified five different *lpnA* sequence types (ST1 to ST5; Fig. 1). Among the five sequence types there were14 polymorphic positions. Hereby, ST1 and ST2, were most similar, with only one nucleotide difference. The largest differences were between ST5 andST1 (12 nucleotide differences) and ST5 andST4 (10 nucleotide differences).

Three *lpnA* sequence types ST1, ST2 and ST4 were identified only in ticks, whereas ST3 was detected in ticks as well as in 3 *A. sylvaticus* (AR1MM1, AR1MM2 and GU1MM1). ST5 was detected only in one *A. sylvaticus* (AR1MM2). This was the same animal in which the *F. hispaniensis*-like sequence was also detected. ST4 was found only in questing *D. reticulatus* and *I. hexagonus* from the Basque Country and Portugal, respectively, and was identical to an *lpnA* sequence previously detected in Portuguese ticks (PoTiEF1; GenBank accession GU113085). The *lpnA* sequence types detected in *A. sylvaticus* (ST3 and ST5) grouped together and separated from the sequence types detected in a wide variety of ticks (Fig. 1).

Among the *F. tularensis* subsp. *holarctica* positive ticks and animals from Spain and Portugal, the *lpnA* sequences (EU315911; EU315912; EU315916; EU315913; EU315915; EU 315914; KJ477083; KJ477082; KJ689454; KJ734992; KJ477079; KJ477081; KJ477084; FJ51540) showed 100% identity to each other and grouped with the previously reported sequences from the area (GenBank accessions AY219238; 13, 14) (Fig. 1).

4. Discussion

In this study, we demonstrated that FLEs are present in a wide range of tick species across the Iberian Peninsula and found evidence that FLEs also appear to be present in free-living wild animals in the same region. These data suggest that small mammals may be potential hosts for FLEs. In addition, we detected the presence of an *F. hispaniensis*-like sequence in small mammals and we confirm the presence of *F. tularensis* subsp. *holarctica*, in both ticks and small mammals, in the Iberian Peninsula.

In previous studies, the percentage of patients in Europe who developed clinical tularemia after a tick bite varied between 12.8% (Slovakia) and 26% (France) (http://www.invs.sante.fr/pmb/invs/(id)/PMB_11678). A few sporadic tick-borne cases have been described in Spain (Teijo-Núñez et al., 2006), consistent with the

Table 2
Ticks collected from animals and positives to *Francisella* sp.

	Madrid					Basque Country					Portugal				
	N	Animal species	Positive FLE (%)	FLE (*lpnA* sequence type)	Positive *F. tularensis* subsp. *holarctica* (%)[a]	*N*	Animal species	Positive FLE (%)	FLE (*lpnA* sequence type)	Positive *F. tularensis* subsp. *holarctica* (%)[a]	*N*	Animal species	Positive FLE (%)	FLE (*lpnA* sequence type)	Positive *F. tularensis* subsp. *holarctica* (%)
Hyalomma lusitanicum	94	Cattle, deer, dog, hedgehog, pig, sheep, wild boar	65 (67.0)	ST1	0	0					3	Rabbit	0		0
Ixodes hexagonus	0					0					13	Dog	0		0
Rhipicephalus pusillus	61	Cat, cattle, common buzzard, dog, fox, hare, hedgehog, rabbit	14 (22.9)	ST1, ST2, ST3	0	0									
Dermacentor marginatus	83	Cattle, deer, horse, wild boar	7 (8.4)	ST1, ST3	2 (2.4)	0									
Rhipicephalus sanguineus	146	Beech marten, cat, hare, hedgehog, sheep	7 (4.5)	ST1, ST3	1 (0.6)	0					10	Rabbit, dog	0		0
Rhipicephalus bursa	40	Cattle	2 (5.0)	ST1	0	0									
Haemaphysalis hispanica	5	Rabbit	0		0	0									
Hyalomma marginatum	13	Cattle, horse	0		0	0					1	Rabbit	0		0
Amblyomma latum	1	Snake	0		0	0									
Total	443		95 (21.4)		3 (0.6)	0					27		0		0

[a] Ticks were considered *F. tularensis* subsp. *holarctica* PCR positive by testing lpnA.

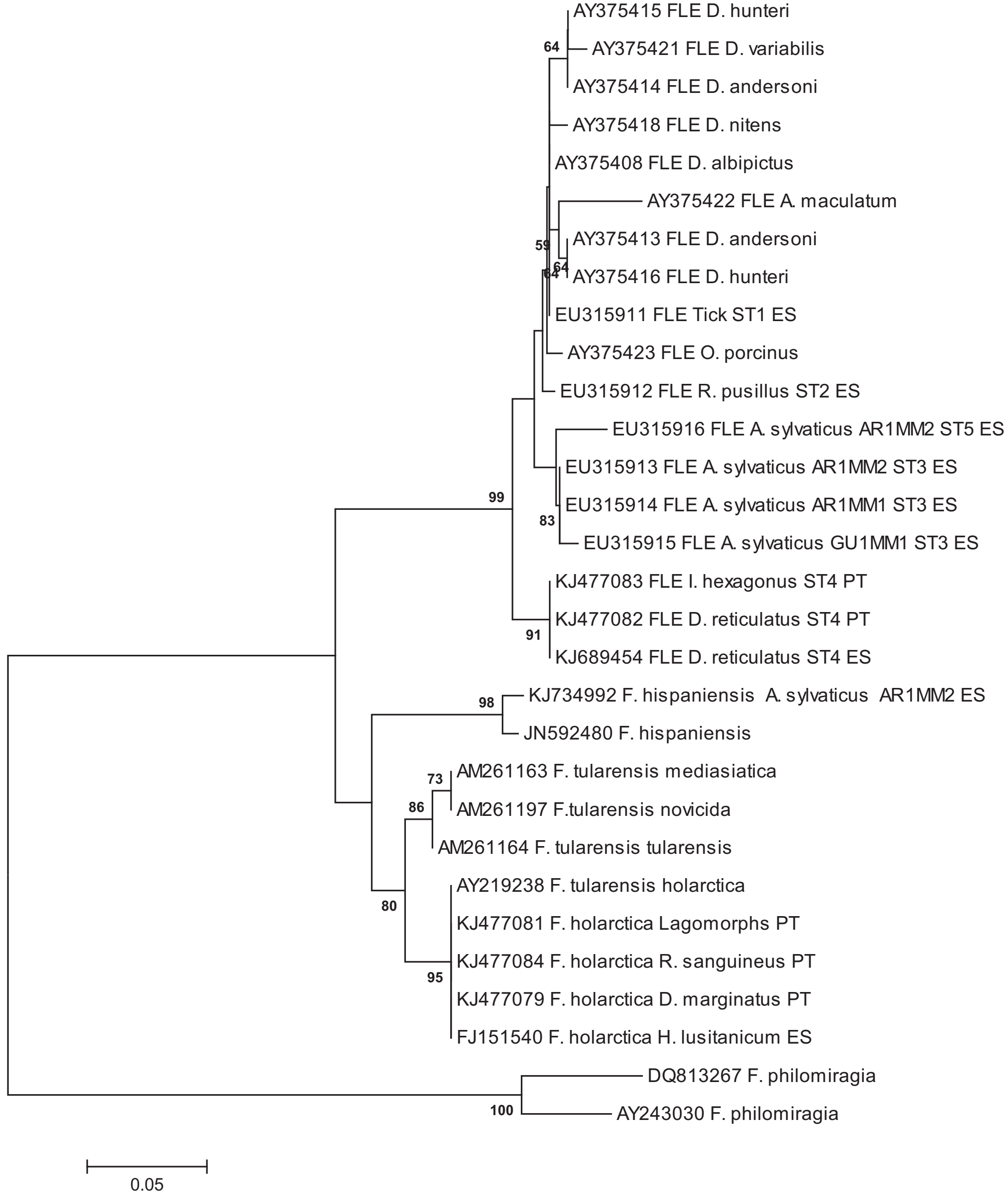


Fig. 1. Phylogenetic analysis of partial *lpnA* sequences (215 bp) of *F. tularensis holarctica* and FLEs detected in Portugal and Spain as compared with other sequences from GenBank. The phylogeny was inferred by neighbor-joining using Mega software version 6. Bootstrap values over 60% obtained from 1000 replicate trees are shown at the nodes. Sequences detected in Portugal are identified with _PT and in Spain with _ES.

identification of *F. tularensis* in tick species that bite humans. Although tularemia is on the list of notifiable diseases, no tick-borne cases have been declared in Portugal thus far, possibly due to a low clinical awareness. However, a seroepidemiological study performed in the Évora region in1999 detected 2.5% seropositivity in blood samples collected from hunters (Núncio, 2002). The positive lagomorph identified in this study were all collected in same area, implying that the agent has been circulating in this region since at least 1999.

FLEs were previously detected in *D. reticulatus* in the Iberian Peninsula (Escudero et al., 2008; de Carvalho et al., 2011,) as well as in different countries throughout Europe including Hungary (Sréter-Lancz et al., 2009; Ivanov et al., 2011; Kreizinger et al., 2013; Wójcik-Fatla et al., 2015) and Serbia (Tomanović et al., 2013). In this study we detected FLEs in *D. reticulatus* as well as additional species of questing (*H. lusitanicum*, *D. reticulatus*, and *I. hexagonus*) and feeding ticks (*D. marginatus*, *H. lusitanicum*, *R. bursa*, *R. pusillus* and *R. sanguineus*), indicating that FLEs are widely distributed in a number of different tick species throughout the Iberian Peninsula. Three of the tick species harboring FLEs, *D. reticulatus*, *H. lusitanicum*, *R. sanguineus*, bite humans. *Dermacentor* species are anthropophilic and associated with transmission of *F. tularensis* to both humans and animals. *H. lusitanicum* is the predominant tick species in the south and central areas of the Iberian Peninsula and several tick-borne pathogens, including *F. tularensis*, have been detected in this tick species (Toledo et al., 2009). It is frequently associated with cattle and occasionally parasitizes humans. *R. sanguineus*, is present in peridomestic areas; it ismostly associated with domestic dogs andplays an important role in the transmission of infectious diseases in the Iberian Peninsula, particularly boutonneuse fever (Santos-Silva et al., 2011).

The variability observed among FLE *lpnA* sequence types indicates that FLEs, present in both ticks and animals, are a heterogenic group of organisms. The taxonomy of this group seems to be rather

Table 3
Positives to *Francisella tularensis* subsp. *holarctica* (Fth), *Francisella hispaniensis-like* (Fh) and *Francisella*-like endosymbionts (FLE) detected in small mammals and lagomorphs analyzed.

Tick species	Madrid				Basque Country				Portugal				
	N	Positive (%)[a,b]	Fth[c]	FLE[c]	*N*	Positive (%)	Fth	FLE	*N*	Positive (%)[d,e]	Fth	FLE	FLEs *lpnA* sequence types
Apodemus sylvaticus	22	3 (13.6)	0	3	162	0			0				ST3, ST5
Apodemus flavicollis	0				3	0			0				
Myodes glareolus	0				16	0			0				
Sorex coronatus	0				14	0			0				
Crocidura russula	3	0			6	0			0				
Mus musculus	1	0			28	0			0				
Talpa europaea	0				24	0			0				
Microtus duodecimcostatus	1	0			0				0				
Oryctolagus cuniculus	0				0				79	5 (6.3)	4 (5.1)		
Lepus europaeus	0				0				14	2 (14.3)	2 (14.3)		
Total	27	3 (11.1)	0	3 (100)	253	0			93	7 (7.5)	6 (6.4)		

[a] One of the three positive specimens was positive for both FLE and *F. hispaniensis-like sequences*.
[b] Samples were considered *Francisella* sp. PCR positive by testing the *lpn*A gene.
[c] Samples were considered *F. tularensis* or FLE positive by *lpn*A sequencing. Samples were also analyzed by *sdh*A amplification but no sequence was obtained.
[d] Samples were considered *F. tularensis* subsp. *holarctica* by testing RT- TypeB and Ft-M19.
[e] One of the *Francisella* positives could only be identified to the species level (*Francisella* sp.).

complex, and our preliminary evidence suggests a possible association with distinct hosts. The ST1 sequence type was detected only in ticks (*D. marginatus*, *H. lusitanicum*, *R. pusillus*, *R. sanguineus* and *R. bursa*) and is most closely related to the previously described FLEs in *Dermacentor* (DAS and DAV) and *Ornithodoros* species (Niebylski et al., 1997) (Fig. 1). ST2 and ST4 were also only detected in ticks, and in the case of ST4, within the same tick species (*D. reticulatus*) from geographically distant areas (Portugal and the Basque Country). The ST3was detected in both ticks and *A. sylvaticus*, whereas ST5 was detected only in *A. sylvaticus.* Interestingly, only one ST type per tick species was identified whereas multiple ST types were identified in *A. sylvaticus*. Collectively, these results suggest that there may be ST type host specificity. We note that tick infections may also be a result of feeding on an infected animal.

Interestingly, ST3 sequence types, with 100% sequence identity, were identified in both small mammals and ticks, suggesting that transmission of FLEs, from ticks to mammals, may occur in nature. Direct transmission by ticks may be possible if the FLE is present in salivary glands or alternatively if ticks are ingested during animal grooming. Moreover, larvae of *D. marginatus* usually feed on rodents as primary hosts. Previous reports demonstrated pathogenicity of FLEs from *D. andersoni* and soft ticks when inoculated intraperitoneally into guinea pigs and hamsters (Burgdorfer et al., 1973; Niebylski et al., 1997). Given the identification of FLEs in animals in this study, it will be important to address whether transmission (either direct or indirect) occurs in laboratory studies in order to assess the potential public health risk.

Ticks are not only vectors for the transmission of *F. tularensis*, but also can potentially maintain the organism in nature between epizootics (Ellis et al., 2002). In this study, the causative agent of tularemia was identified in *D. marginatus*, *D. reticulatus*, *H. lusitanicum*, *I. ricinus* and *R. sanguineus*, tick species previously shown to be positive for *F. tularensis* (de Carvalho et al., 2007; Toledo et al., 2009). The prevalence of *F. tularensis* subsp. *holarctica* was higher in both *I. ricinus* (25%) and *R. sanguineus* (25.7%) in this study as compared with previous data (de Carvalho et al., 2007). This could be the result of bias due to the selection of collection sites with a high probability of circulating vector borne agents and requires further analysis.

Our data indicates that *F. tularensis* is present in ticks and lagomorphs in Portugal and that tularemia should be considered after tick bite or hunting and skinning lagomorphs. The identification of *F. tularensis* positive lagomorphs suggests they may play a role in maintenance of *F. tularensis* in Portugal, as occurs in other endemic countries. The prevalence of *F. tularensis* subsp. *holarctica* in *L. europaeus* in this study (14.3%) is higher than previously reported for seroprevalence in European brown hares (4.9–6.9%). The identification of *F. tularensis* subsp. *holarctica* in the European rabbit, *O. cuniculus*, is of interest, particularly given the absence of readily identifiable macroscopic tissue lesions in tissue specimens from these animals (Gyuranecz et al., 2010). Further pathological investigations will be necessary to clarify the importance of *O. cuniculus* as pertains to maintenance of tularemia.

In this study we demonstrated by DNA detection the presence of an *F. hispaniensis*-like organism in a free-living small mammal. It is interesting to note that this positive animal was detected in Spain, where *F. hispaniensis* was first described in a severely ill patient (Escudero et al., 2008). These results provide new insights for further studies, including a better understanding of the geographical distribution and public health relevance of this *Francisella* species.

Disclosure statement

No competing financial interests exist.

Acknowledgements

We would like to thank to the REVIVE Program for the help on ticks collection. This work was supported by Fundação para a Ciência e Tecnologia (FCT) project: PTDC/SAU-ESA/104947/2008 and a scholarship (SFRH/BD/79225/2011); and also by the Fondo de Investigation Sanitaria (PI10/00165) and Instituto de Salud Carlos III (grant EM03/06).

References

Anda, P., Segura del Pozo, J., Díaz García, J.M., Escudero, R., García Peña, F.J., López Velasco, M.C., Sellek, R.E., Jiménez Chillarón, M.R., Sánchez Serrano, L.P., Martínez Navarro, J.F., 2001. Waterborne outbreak of tularemia associated with crayfish fishing. Emerg. Infect. Dis. 7, 575–582.

Barandika, J.F., Olmeda, S.A., Casado-Nistal, M.A., Hurtado, A., Juste, R.A., Valcarcel, F., Anda, P., Garcia-Perez, A.L., 2011. Differences in questing tick species distribution between Atlantic and continental climate regions in Spain. J. Med. Entomol. 48, 13–19.

Barns, S.M., Grow, C.C., Okinaka, R.T., Keim, P., Kuske, C.R., 2005. Detection of diverse new *Francisella*-like bacteria in environmental samples. Appl. Environ. Microbiol. 71, 5494–5500.

Berrada, Z.L., Telford 3rd., S.R., 2010. Diversity of *Francisella* species in environmental samples from Martha's Vineyard, Massachusetts. Microb. Ecol. 59, 277–283.

Byström, M., Böcher, S., Magnusson, A., Prag, J., Johansson, A., 2005. Tularemia in Denmark: identification of a *Francisella tularensis* subsp. holarctica strain by real-time PCR and high-resolution typing by multiple-locus variable-number tandem repeat analysis. J. Clin. Microbiol. 43 (10), 5355–5358.

Burgdorfer, W., Brinton, L.P., Hughes, L.E., 1973. Isolation and characterization of symbionts from the Rocky Mountain wood tick. *Dermacentor andersoni*. J. Invertebr. Pathol. 22, 424–434.

de Carvalho, I.L., Escudero, R., Garcia-Amil, C., Falcão, H., Anda, P., Núncio, M.S., 2007. *Francisella tularensis*, Portugal. Emerg. Infect. Dis. 13, 666–667.

de Carvalho, I.L., Santos, N., Soares, Soares, T., Zé-Zé, L., Núncio, M.S., 2011. *Francisella*-like endosymbiont in *Dermacentor reticulatus* collected in Portugal. Vector Borne Zoonotic Dis. 11, 185–188.

Dennis, D.T., Inglesby, T.V., Henderson, D.A., Bartlett, J.G., Ascher, M.S., Eitzen, E., Fine, A.D., Friedlander, A.M., Hauer, J., Layton, M., Lillibridge, S.R., McDade, J.E., Osterholm, M.T., O'Toole, T., Parker, G., Perl, T.M., Russell, P.K., Tonat, K., 2001. Working Group on Civilian Biodefense Tularemia as a biological weapon: medical and public health management. JAMA 285, 2763–2773.

Eiros Bouza, J.M., Rodríguez-Torres, A., 1998. Tularemia. Rev. Clin. Esp. 198, 785–788.

Ellis, J., Oyston, P.C., Green, M., Titball, R.W., 2002. Tularemia. Clin. Microbiol. Rev. 15 (4), 631–646.

Escudero, R., Toledo, A., Gil, H., Kovácsová, K., Rodríguez-Vargas, M., Jado, I., García-Amil, C., Lobo, B., Bhide, M., Anda, P., 2008. Molecular method for discrimination between *Francisella tularensis* and *Francisella*-like endosymbionts. J. Clin. Microbiol. 46, 3139–3143.

Franke, J., Fritzsch, J., Tomaso, H., Straube, E., Dorn, W., Hildebrandt, A., 2010. Coexistence of pathogens in host-seeking and feeding ticks within a single natural habitat in Central Germany. Appl. Environ. Microbiol. 76, 6829–6836.

Gyuranecz, M., Szeredi, L., Makrai, L., Fodor, L., Mészáros, A.R., Szépe, B., Füleki, M., Erdélyi, K., 2010. Tularemia of European Brown Hare (*Lepus europaeus*): a pathological, histopathological and immunohistochemical study. Vet. Pathol. 47 (5), 958–963.

Gyuranecz, M., Rigó, K., Dán, A., Földvári, G., Makrai, L., Dénes, B., Fodor, L., Majoros, G., Tirják, L., Erdélyi, K., 2011. Investigation of the ecology of *Francisella tularensis* during an inter-epizootic period. Vector Borne Zoonotic Dis. 11, 1031–1035.

Huber, B., Escudero, R., Busse, H.J., Seibold, E., Scholz, H.C., Anda, P., Kämpfer, P., Splettstoesser, W.D., 2010. Description of *Francisella hispaniensis* sp. nov., isolated from human blood, reclassification of *Francisella novicida* (Larson et al., 1955) Olsufiev et al., 1959 as *Francisella tularensis* subsp. *novicida* comb. nov. and emended description of the genus Francisella. Int. J. Syst. Evol. Microbiol. 60, 1887–1896.

Ivanov, I.N., Mitkova, N., Reye, A.L., Hübschen, J.M., Vatcheva-Dobrevska, R.S., Dobreva, E.G., Kantardjiev, T.V., Muller, C.P., 2011. Detection of new *Francisella*-like tick endosymbionts in *Hyalomma* spp. and *Rhipicephalus* spp. (Acari: Ixodidae) from Bulgaria. Appl. Environ. Microbiol. 77, 5562–5565.

Kreizinger, Z., Hornok, S., Dán, A., Hresko, S., Makrai, L., Magyar, T., Bhide, M., Erdélyi, K., Hofmann-Lehmann, R., Gyuranecz, M., 2013. Prevalence of *Francisella tularensis* and *Francisella*-like endosymbionts in the tick population of Hungary and the genetic variability of *Francisella*-like agents. Vector Borne Zoonotic Dis. 13, 160–163.

Kugeler, K.J., Pappert, R., Zhou, Y., Petersen, J.M., 2006. Real-time PCR for *Francisella tularensis* types A and B. Emerg. Infect. Dis. 12, 1799–1801.

MacDonald, D.W., Barrett, P., 1993. Collins Field Guide: Mammals of Britain and Europe. Collins, London, pp. 312 p.

Machado-Ferreira, E., Piesman, J., Zeidner, N.S., Soares, C.A., 2009. *Francisella*-like endosymbiont DNA and *Francisella tularensis* virulence-related genes in Brazilian ticks (Acari: Ixodidae). J. Med. Entomol. 46, 369–374.

Manilla, G., 1998. Acari Ixodida. Fauna díltalia. Edizioni Calderini, Bologna, Italy.

Niebylski, M.L., Peacock, M.G., Fischer, E.R., Porcella, S.F., Schwan, T.G., 1997. Characterization of an endosymbiont infecting wood ticks, *Dermacentor andersoni*, as a member of the genus *Francisella*. Appl. Environ. Microbiol. 63, 3933–3940.

Núncio, M.S., 2002. Contribuição para o estudo de borrélias e borreliose de Lyme em Portugal em Portugal. Report for PhD in Biology. Science Faculty, Universidade de Lisboa.

Peel, M.C., Finlayson, B.L., McMahon, T.A., 2007. Updated world map of the Köppen-Geiger climate classification. Hydrol. Earth Syst. Sci. 11, 1633–1644.

Santos-Silva, M.M., Beati, L., Santos, A.S., De Sousa, R., Núncio, M.S., Melo, P., Santos-Reis, M., Fonseca, C., Formosinho, P., Vilela, C., Bacellar, F., 2011. The hard-tick fauna of mainland Portugal (Acari: Ixodidae): an update on geographical distribution and known associations with hosts and pathogens. Exp. Appl. Acarol. 55, 85–121.

Scoles, G.A., 2004. Phylogenetic analysis of the *Francisella*-like endosymbionts of Dermacentor ticks. J. Med. Entomol. 41, 277–286.

Sjödin, A., Svensson, K., Ohrman, C., Ahlinder, J., Lindgren, P., Duodu, S., Johansson, A., Colquhoun, D.J., Larsson, P., Forsman, M., 2012. Genome characterization of the genus *Francisella* reveals insight into similar evolutionary paths in pathogens of mammals and fish. BMC Genom. 13, 268.

Sjöstedt, A.B., 2005. *Francisella*. The Proteobacteria, Part B. In: Brenner, D.J., Staley, J.T., Garrity, G.M. (Eds.), Bergey's Manual of Systematic Bacteriology. Springer, New York, N.Y, pp. 200–210 (vol. 2, 2nd ed., vol. 2).

Sréter-Lancz, Z., Széll, Z., Sréter, T., Márialigeti, K., 2009. Detection of a novel *Francisella* in *Dermacentor reticulatus*: a need for careful evaluation of PCR-based identification of *Francisella tularensis* in Eurasian ticks. Vector Borne Zoonotic Dis. 9, 123–126.

Sun, L.V., Scoles, G.A., Fish, D., O'Neill, S.L., 2000. Francisella-like endosymbionts of ticks. J. Invertebr. Pathol. 76, 301–303 (Surveill 12: E071108).

Szigeti, A., Kreizinger, Z., Hornok, S., Abichu, G., Gyuranecz, M., 2014. Detection of *Francisella*-like endosymbiont in *Hyalomma rufipes* from Ethiopia. Ticks Tick Borne Dis. 5, 818–820.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol. Biol. Evol. 30, 2725–2729.

Teijo-Núñez, C., Escudero-Nieto, R., Fernández-Natal, M.I., Anda-Fernández, P., 2006. New case of autochthonous tick-borne tularemia. Enferm. Infecc. Microbiol. Clin. 24 (8), 533.

Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673–4680.

Toledo, A., Olmeda, A.S., Escudero, R., Jado, I., Valcárcel, F., Casado-Nistal, M.A., Rodríguez-Vargas, M., Gil, H., Anda, P., 2009. Tick-borne zoonotic bacteria in ticks collected from central Spain. Am. J. Trop. Med. Hyg. 81, 67–74.

Tomanović, S., Chochlakis, D., Radulović, Z., Milutinović, M., Cakić, S., Mihaljica, D., Tselentis, Y., Psaroulaki, A., 2013. Analysis of pathogen co-occurrence in host-seeking adult hard ticks from Serbia. Exp. Appl. Acarol. 59 (3), 367–376.

Versage, J.L., Severin, D.D., Chu, M.C., Petersen, J.M., 2003. Development of a multitarget real-time TaqMan PCR assay for enhanced detection of *Francisella tularensis* in complex specimens. J. Clin. Microbiol. 41, 5492–5499.

Wójcik-Fatla, A., Zając, V., Sawczyn, A., Cisak, E., Sroka, J., Dutkiewicz, J., 2015. Occurrence of *Francisella* spp. in *Dermacentor reticulatus* and *Ixodes ricinus* ticks collected in eastern Poland.

Chapter VIII

Francisella-like endosymbionts

1. Introduction

The first tick endosymbiont was isolated in 1961, the bacterium "*Wolbachia persica*" (Suitor and Weiss, 1961). Since then, a wide range of symbionts, such as *Coxiella*-like, *Francisella*-like or *Rickettsia*-like, have been detected in several tick species (Liu et al., 2013).

Symbionts affect development, nutrition, reproduction, speciation, defence against natural enemies and environmental stress, and immunity of their hosts (reviewed in (Dale and Moran, 2006).

It is proposed that ticks endosymbionts provide their host essential nutrients that they are unable to obtain from the vertebrate blood (Gerhart et al., 2016). For instance, *Coxiella*-like organisms have a possible role in nutrition, especially in the nitrogen metabolism, by potentially provisioning their hosts with essential nutriments (Lalzar et al., 2014). Similarly, it seems that some FLEs may synthesize important amino acids such as cysteine, threonine, tyrosine, tryptophan, phenylalanine, and serine from pyruvate, and can metabolize glutamate, glutamine, and asparagine into ATP (Gehart et al., 2016). The synthesis of glutamine from glutamic acid and ammonia, allows for the recycling of cellular ammonia waste to useful products (Gehart et al., 2016).

1.1. Summary on symbionts classification and transmission patterns

Symbionts are traditionally classified as obligate (primary) or facultative (secondary) regarding the degree of dependence between the host and the symbiont (Dale and Moran, 2006)(Chaves et al., 2009). In addition, arthropod symbionts present tissue tropism in relation to the nature of the association and the mode of transmission between host generations (Lalzar et al., 2014). Obligate symbionts have been shown to descend from ancient and specializes associations (Dale and Moran, 2006). They are often restricted to specialized tissues or cells (Chaves et al., 2009) and beneficial or even essential for the survival of the host to which they provide nutrients (Dale and Moran, 2006)(Moran et al., 2008). Usually obligate symbionts are vertically transmitted from mother to offspring via infection of eggs or embryos (Dale and Moran, 2006)(Chaves et al., 2009). Facultative symbionts are usually not restricted to specific tissues (Liu et al., 2013), seem to have established more recent associations with their hosts (Dale and Moran, 2006)(Chaves et al., 2009), to which they are often unessential (Liu et al., 2013). These associations can be deleterious or beneficial (e.g. protection against aggressors or stress (Moran et al., 2008)) (Dale and Moran, 2006). Facultative symbionts can be transmitted either vertically or horizontally between the same or different species (Liu et al., 2013). Commonly, multiple symbionts can coexist in the same host individual, most often

one or two primary symbionts and one or two secondary endosymbionts (Dale and Moran, 2006), which interact with each other, and co-regulate the biological processes of the host (Liu et al., 2013).

Co-infection of ticks with multiple endosymbionts have been reported (Liu et al., 2013) that may have evolved under complex multi-specific interactions (Clay et al., 2008), suggesting that endosymbiotic systems can be dynamic across tick lineages (Duron et al., 2015).

1.2. FLEs transmission patterns

The *Francisella*-like endosymbionts (FLEs) are amongst the most common ticks symbionts (Liu et al., 2016). Although the phylogenies of FLEs and their tick hosts are parallel at the genus level, they are unresolved at the species level, as observed for the *Dermacentor* FLE (Scoles, 2004). In addition, mixed infections with two FLEs with divergent 17-kDa lipoprotein gene sequences were reported. It is therefore unlikely that FLEs have co-speciated closely with their tick hosts as symbiont sequences do not always parallel the tick phylogeny (Page, 2003)(Scoles, 2004). The fact that there is little sign of co-speciation, could indicate that the association between FLEs and their host ticks is of a relatively recent origin (Scoles, 2004). In their tick hosts, FLEs are vertically transmitted (Baldridge et al., 2009)(Liu et al., 2016). The observation of perfect vertical transmission of some FLEs suggest that they are important symbionts and may contribute to the fitness of their tick hosts (Baldridge et al., 2009)(Liu et al., 2016). While some FLEs were found to be confined to ovarian tissues and Malpighian tubules, others have also been detected in the salivary glands aside (Goethert and Telford, 2005) and even in the midgut of ticks (Liu et al., 2016), suggesting that, despite crucial symbionts, FLEs probably do not have specific tissue tropism (Liu et al., 2016).

1.3 Heritable symbiosis

As reviewed by Bennett and Moran (2015), bacteria with high metabolic proficiency are known to replace ancient endosymbionts with reduced metabolic competence. Obligate symbiosis can limit the ecological range of hosts, reduce population sizes, or even cause extinction of some symbiont-dependent host lineages along with their symbiont. The fitness interests of obligate heritable symbionts are distinct from those of their hosts, leading to selfish tendencies. When a symbiont is required for development, hosts may become locked in, even when the original symbiotic benefit is reduced or eliminated due to changing ecological conditions or deterioration of symbiont functionality. Once a host lineage has progressed down the irreversible path into obligate "rabbit

hole" symbiosis, there is little opportunity to exit. Usually, escaping from degenerate partners involves supplement or replace the ancient symbiont with a new one. A likely driver for addition of a new symbiont is the degradation of functions in an ancient one, so that the new symbiont can replace or supplement functions that are lost or inefficient in the older partner. A recent study involving sequencing of the genome of a FLE (FLE-Am) present in the Gulf Coast tick (*Amblyomma maculatum*) indicate that FLE-Am transformed recently into an endosymbiont and likely replaced an ancestral endosymbiont with degraded functionality, allowing *A. maculatum* to escape the "symbiosis rabbit hole" (Gehart et al., 2016).

As FLE-Am was shown to enclose pseudogenized versions of several virulence genes, including genes for a Type VI Secretion System present on a pathogenicity island in *F. tularensis* and for Type 4 pili that are critical to vertebrate infection, it was proposed that the ancestor of FLE-Am was most likely a vertebrate pathogen containing functional versions of virulence genes, despite no clear examples of this process have been documented (Gehart et al., 2016). By contrast, the evolutionary transformation of a maternally inherited endosymbiont of ticks into a specialized and virulent pathogen of vertebrates was shown for the *Rickettsia* genus (Weller et al., 1998) and recently also for *Coxiella burnetii* (Duron et al., 2015). This rare evolutionary transition observed within the *Coxiella* genus required evolving metabolic adaptations that led to the emergence of a vertebrate infectious disease (Duron et al., 2015). Although identifying the evolutionary processes that transform symbiotic bacteria into emerging pathogens requires further exploration, these may include spontaneous genetic mutations and the transfer and integration of virulence genes from a co-infecting pathogen (Duron, 2014)(Nikoh et al., 2014)(Bennet et al., 2015)(Duron et al., 2015).

The genome reduction in FLE-Am resulting in the absence of intact secretion and effector gene systems suggests that it may be avirulent to humans despite its presence in the salivary glands and saliva of *A. maculatum* (Gehart et al., 2016).

Futher research is now needed to assess the potential of different endosymbionts, such as *Coxiella*-like or *Francisella*-like organisms, to infect vertebrates.

1.4. Methods for discriminating between *Francisella tularensis* and FLEs

FLEs and *F. tularensis* species belong to different phylogenetic clades (Scoles, 2004) but their high similarity as well as the presence of FLEs in tick species associated with the transmission of tularaemia makes absolutely imperative the accurate identification of *F. tularensis* (Kugeler et al., 2005)(Escudero et al., 2008). FLEs cross-react with most of the techniques used for the detection of

F. tularensis potentially leading to misidentification and misinterpretation (Kugeler et al., 2005) and methods with specific targets must be used. As referred in General Introduction in the review article on tularaemia and in the specific introduction of chapter VI "Laboratorial diagnosis of *F. tularensis*", a multi-target PCR assay has been described for the detection of *F. tularensis* in a wide variety of field and environmental samples and is based on the amplification of four sequences that have proven to discriminate *Francisella* spp., namely the *ISFtu*2 element, and the *23 kDa*, *tul*4 and *fop*A genes (Versage et al., 2003). In addition, a highly sensitive and specific molecular method for the differentiation of *F. tularensis* and FLEs was developed based on the narrow nucleotide variability found between the lipoprotein-coding *lpn*A gene of *Francisella* pathogens and that of FLEs (Escudero et al., 2008). The method included the amplification of a 233-bp fragment on a variable region of *lpn*A that and further hybridization with specific probes by reverse line blotting (RLB) (Escudero et al., 2008). Also, the real time multitarget TaqMan PCR assay (*ISFtu*2, *tul*4, and *igl*C) can discriminate *F. tularensis* from *Francisella*-like tick endosymbionts of *D. variabilis* and *D. occidentalis* and may be useful in laboratories that screen these species for *F. tularensis* (Kugeler et al., 2005).

Study 12

What role do *Francisella*-like endosymbionts play in the transmission of vector-borne diseases?

Francisella-like endosymbiont and pathogenic *Rickettsia aeschlimannii* co-infection in *Hyalomma marginatum*

Carina L. Carvalho[1,2],Rita de Sousa[2], Margarida M. Santos-Silva[2], Ana S. Santos[2], Maria S. Núncio[2] and Isabel Lopes de Carvalho[2]

[1]Instituto de Ciências Agrárias e Ambientais Mediterrânicas (ICAAM), Universidade de Évora, Évora, Portugal; [2]Centro de Estudos de Vectores e Doenças Infecciosas, Instituto Nacional de Saúde Doutor Ricardo Jorge (INSARJ), Águas de Moura, Portugal

Manuscript in preparation

1. Abstract

Francisella-like endosymbionts (FLEs) are worldwide distributed and have been detected in several species of hard and soft ticks. Recently, they were also reported in small free-living mammals in Europe. FLEs' pathogenicity to humans is unknown and their potential effect on vector competency in the transmission of *Francisella tularensis,* or other tick-borne agents, to humans remains undetermined.

A total of 341 ticks belonging to seven species previously tested for *Rickettsia* and *Borrelia burgdorferi* s.l., were analyzed by conventional PCR targeting *tul*4 gene for *F. tularensis* Ticks were collected from the vegetation (n=108) and from different mammalian hosts (n=233).

All ticks tested negative for *F. tularensis* but nucleotide sequences identical to FLEs were obtained in 2.34% of the questing ticks, including *Dermacentor reticulatus* (1.76%), *Hyalomma marginatum* (0.29%) and *H. lusitanicum* (0.29%). Furthermore, one *H. marginatum* tick was detected with FLEs (0.29%) co-infected with *Rickettsia aeschlimannii*, a spotted fever group pathogen.

2. Body of the manuscript

Francisella tularensis is a facultative intracellular pathogen, belonging to gamma (γ)-Proteobacteria, responsible for a highly infectious zoonotic disease known as tularaemia. *F. tularensis* is formally divided into three subspecies, namely *F. tularensis* subsp. *tularensis*, *F. tularensis* subsp. *holarctica* and *F. tularensis* subsp. *mediasiatica* (Vogler et al., 2009).

In nature, *F. tularensis* has been detected in a broad host range comprising several mammalian species including lagomorphs and rodents which are considered the main reservoirs for this pathogen, but also in birds, amphibians, fish and even invertebrates (Mörner, 1992)(Gyuranecz et al., 2010). Humans commonly acquire the infection either by direct contact with infected animals or through the bite of arthropod vectors such as ticks and mosquitoes (Mörner, 1992)(Petersen et al., 2009)(Telford and Goethert, 2010).

Francisella-like endosymbionts (FLEs) are among the most common tick symbionts (Liu et al., 2016) and have been identified in several hard and soft tick species (Niebylski et al., 1997)(Scoles, 2004)(Escudero et al., 2008)(de Carvalho et al., 2011) (Ivanov et al., 2011)(Dergousoff and Chilton, 2012)(Kreizinger et al., 2013). FLEs have a worldwide distribution having been reported in North America (Scoles, 2004) (Barns et al., 2005)(Kugeler et al., 2005)(Baldridge et al., 2009), Canada (Scoles, 2004)(Baldridge et al., 2009), as well as in European countries such as Spain (Escudero et al., 2008), Portugal (de Carvalho et al., 2011), Hungary (Sréter-Lancz et al., 2009), Serbia (Tomanović et al., 2013) and Bulgaria (Ivanov et al., 2011). They do not grow on cell-free media, limiting their accurate classification (Ivanov et al., 2011), and obscuring the evolutionary relationships among FLEs obtained from different hosts and with the arthropod-borne pathogen *F. tularensis* (Scoles, 2004). The recent comparison of the complete genome of a FLE (FLE-Am) detected in the Gulf

Coast tick (*Amblyomma maculatum*) agrees with the genome evolution from a pathogenic ancestor (Gerhart et al., 2016). This hypothesis is supported by the observations that FLE-Am has undergone minimal genome reduction, but several of its protein-coding genes, including virulence genes, contain inactivating mutations (Gerhart et al., 2016). This may suggest that tick endosymbionts could evolve from mammalian pathogens (Gerhart et al., 2016). The opposite mechanism involving the evolutionary transition from a tick-symbiont to a vertebrate pathogen, is suggested for *Rickettsia* (Weller et al., 1998) and *Coxiella burnetti* (Duron et al., 2015).

The pathogenicity of FLEs to humans as well as their putative effect on vector competency and in the transmission of *F. tularensis* or other tick-borne pathogens is unknown. They have significant nucleotide similarity with *F. tularensis* in the 16S rRNA sequence although they do not belong to the pathogenic *F. tularensis* phylogenetic clade (Scoles, 2004). Differentiation must therefore be accomplished due to the distinct medical implications of the two organisms (Versage et al., 2003)(Escudero et al., 2008).

A total of 341 ticks previously screened for the presence of important tick borne-pathogens causing human disease in Portugal, namely *Rickettsia* and *Borrelia burgdorferi* s.l, were investigated for the presence of *F. tularensis*. Ticks originated from four main geographic regions of Portugal mainland (NUTs II), namely Alentejo, Lisboa e Vale do Tejo (LVT), Norte and Algarve, and were gathered by the Portuguese network for surveillance of arthropod vectors (REVIVE). The specimens were collected between 2011 and 2015 from the vegetation (n=108) using the flagging method, and from mammalian hosts including humans (n=127), dogs (n=8) and deer (n=98). Ticks were morphologically identified as *Dermacentor marginatus* (n=10), *D. reticulatus* (n=19), *Hyalomma marginatum* (n=8), *H. lusitanicum* (n=8), *Ixodes ricinus* (n=266), *Rhipichephalus pusillus* (n=1) *and R. sanguineus* s.l (n=29) (Table 1).

The MagCore Genomic DNA Tissue Kit, cartridge Code 401, RBC (Bioscience Corp.) was used for DNA extraction in ticks collected from humans hosts. For DNA extraction in ticks collected from vegetation and other mammalian hosts, ticks were washed in 70% ethanol and sterile distilled water, dried on sterile paper and subsequently boiled in 25% ammonium hydroxide solution, as described previously by Schouls et al. (1999). Ticks were screened for *F. tularensis* by conventional PCR using primers FT393 and FT642 (Karhukorpi and Karhukorpi, 2001), which amplify a 250nt–long fragment within the coding sequence of gene *tul*4 encoding for the superficial membrane lipoproteins. Nucleotide sequences were assembled using the BioEdit software (Ibis Biosciences, Carlsbad, CA) and submitted to GenBank (MF497787-94).

The previous screening for the presence of *Rickettsia* was performed by targeting the citrate synthase (*gltA*) and outer membrane protein A (*ompA*) and B (*ompB)* as described previously (De Sousa et al. 2006) and for *B. burgdorferi* s.l. by PCR amplification of the 5S (rrf)-23S (rrl) intergenic spacer (Rijpkema et al., 1995).

Overall, 10.3% of the ticks were infected with *Rickettsia*, namely with *R. aeschlimannii* (1.2%), *R. helvetica*, (1.5%), *R. monacensis* (5.3%), *R. slovaca* (0.9%), *R. conorii* (0.3%), *R. massiliae* (0.9%) and *R. raoulti* (0.3%), and 1.76% with *B. burgdorferi* s.l., namely with *B. garinii* (n=1), *B. afzelii* (n=1) and *B. lusitaniae* (n=4). One *I. ricinus* tick (0.3%) was co-infected with *B. lusitaniae* and *R. helvetica*. However, they were all negative for *F. tularensis* by the method described by (Karhukorpi and Karhukorpi, 2001). Although this PCR targets a *Francisella* structural gene (*tul*4), the primers also amplify the homologous region in FLEs' strains. Sequencing analyses of the positive amplicons revealed that the positive samples (8/341, 2.34%) were FLE-positive, none exhibiting similarity with *Francisella* (Table 1).

Six partial *tul*4 gene sequences 200 bp-long (MF497789-94) were obtained from *D. reticulatus* ticks collected from humans (n=2) and dogs (n=4), all showing 100% similarity with the FLE strain EU126640, also obtained from a *D. reticulatus* specimen (FDrH). Despite the reduced sampling size, these results reveal a higher percentage of positivity in ticks collected from dogs (4/8, 50% positives) than from humans (2/11, 18.2% positives), which is expectable due to the higher contact of dogs with environments favorable for ticks.

Among the eight *H. lusitanicum* ticks collected from human hosts that were analysed, only a weak amplicon was generated in one sample. Sequencing was limited to a 100 bp region (accession number MF497788), but blast analysis of this short regions showed 97.2% similarity (difference in one nucleotide) with the FLE strain FLE031 of *H. aegyptum* (HQ705175) and 96.3% (difference in two nucleotides) similarity with FLE strain FLE011 of *H. marginatum* (HQ705174).

Moreover, a 150 bp sequence was also obtained from a *H. marginatum* specimen collected from a human host (accession number MF497787), showing 98.7% of similarity with the FLE strain FLE011 (HQ705174). This detection represents 12.5% (1/8) FLE positivity in the *H. marginatum* ticks investigated. Interestingly, this *H. marginatum* tick was also positive to *R. aeschlimannii*, a pathogenic SFG rickettsia. The human host from which this double-infected *H. marginatum* tick was removed did not present any symptoms at the sampling time neither developed any clinical signs shortly after his medical appointment, as confirmed by the health authorities.

Ticks symbionts are capable of providing some fitness advantages for ticks by co-regulating the biological processes of the host, affecting its development, nutrition, reproduction and speciation, defense and immunity (Dale and Moran, 2006)(Liu et al., 2013)(Liu et al., 2016). As an example *Wolbachia* is a widespread symbiont in arthropods that can protect *Drosophila* and mosquito species against viral infections (Martinez et al., 2014). Recent work on arthropod vectors showed the importance of symbionts on the epidemiology of pathogens (Ryder et al., 2014). A thin line seems to exist between tick symbionts and tick-borne pathogens, as many of the adaptations that might potentiate tick-borne transmission of a pathogen may also favor the establishment of a symbiotic relation (Scoles, 2004). In addition, mixtures of symbionts (Noda et al., 1997)(Ahantarig et

al., 2013) and co-infections with multiple symbionts (Liu et al., 2013) or pathogens (Moutailler et al., 2016)(Raileanu et al., 2017) or with symbionts and pathogenic bacteria have been reported in ticks (Moutailler et al., 2016).

H. lusitanicum is one of most abundant tick species found in the south and central regions of the Iberian Peninsula. Several tick-borne pathogens, including *F. tularensis*, have been detected in this species (Toledo et al., 2009), frequently associated with cattle and that occasionally parasitizes humans (Santos-Silva., et al., 2011). Although the infection of *Hyalomma* ticks with FLEs has been described before, namely in *H. marginatum*, *H. aegyptum* (Ivanov et al., 2011) and *H. lusitanicum* (Lopes de Carvalho et al., 2016), to our knowledge, the mixed infection with FLEs and *R. aeschlimannii* is a novelty, at least in this tick genus. The co-infection of this *H. marginatum* tick may have occurred during the immature stage feeding on a host infected with both bacteria.

The co-infection of ticks with FLEs and a *Rickettsia* species was previously described in ticks of the *Dermacentor* genus (Niebylski et al., 1997)(Scoles, 2004) (Dergousoff and Chilton, 2012). *Dermacentor* species are known to be anthropophilic and are associated with transmission of *F. tularensis* to humans and animals, even in Portugal (de Carvalho et al., 2007). The most relevant finding of this study was the detection of a co-infection with a FLE strain and *R. aeschlimannii* in *H. marginatum* ticks collected from a human host. *R. aeschlimanni* was isolated for the first time in *H. marginatum* in 1997 (Beati et al., 1997) and was recognized as a pathogenic agent and included in the SFG in 2002 (Raoult et al., 2002).

The role of FLEs and most tick endosymbionts remains poorly understood as only a few bacteria and tick species have been studied. Whether FLEs modulate tick vector competence in the transmission of *F. tularensis* (Goethert and Telford, 2005) or other pathogens such as *Rickettsia* to humans or other animals remains unknown (Scoles, 2004)(Dergousoff and Chilton, 2012). For instance, *Rickettsia*-like symbionts are suggested to affect tick physiology, population dynamics and transmission of pathogenic *rickettsia.* FLEs are intracellular and have been mainly found in reproductive tissues of female ticks, being transmitted transovarially. Apparently, the presence of FLEs do not inhibit the vertical transmission of distantly related organisms such as *Rickettsia* spp. (there is a report of ovary cells containing both FLE and *Rickettsia* (Niebylski et al., 1997) or *Anaplasma phagocytophilum* (Baldridge et al., 2009). Also, the presence of one FLE strain does not impede infection with either a second closely related symbiont or with a second symbiont less closely related (Scoles, 2004), nor inhibit the vertical transmission of other FLE strains (Goethert and Telford, 2005) (Dergousoff and Chilton, 2012).

Overall, endosymbionts appear to be harmless to mammals and there is no evidence of FLEs' pathogenicity to humans (Scoles, 2004). For instance, the absence of intact secretion and effector gene systems in FLE-Am suggests that it is avirulent to humans despite its presence in the salivary glands and saliva of the tick *A. maculatum* (Gerhart et al., 2016). In addition, some FLEs seem to

lack or at least have a significantly different RD1 (region of difference) sequence, an important molecular marker for the discrimination of pathogenic *F. tularensis* subspecies (Ivanov et al., 2011). Nevertheless, these bacteria have been detected in small free-living mammals in Europe, highlighting the need to clarify the potential transmission of some FLE strains from ticks to mammalian host, if present in the salivary glands or alternatively ingested along with ticks via grooming (Lopes de Carvalho et al., 2016).

As novel FLEs are revealed (such as the *Francisella*-like endosymbiont (FLEs-Hd) detected in *Haemaphysalis doenitzi* (Liu et al., 2016)), understanding the role of these microbial associations in the transmission of tick-borne agents is necessary.

3. Acknowledgements

The authors would like to thank Lígia Chaínho and Teresa Luz for their technical assistance and to REVIVE network. The authors also want to thank to Doctors Ana Claúdia Norte and Joana Alves for tick collection. This study was partially funded by a grant from the Fundação para a Ciência e Tecnologia (FCT) SFRH/79225/2011.

Table 1: Information on the ticks origin, species and collection date. The results obtained during this study are also summarized.

Tick origin	Tick species	Sample size (% of total sample)	Year of collection	Sample size per year (% per tick species)	Testing *F. tularensis*	Testing FLEs	Percentage of FLEs infected ticks % pos (nº samples/ tick specie)	Percentage of co-infected ticks
Human	*D. marginatus*	10 (2.9)	2011	6 (60)	N	N	-	-
			2012	2 (20)	N	N	-	-
			2013	2 (20)	N	N	-	-
	D. reticulatus	11 (3.2)	2014	11 (100)	N	P[a]	0.59% (2/341)	-
	H. marginatum	8 (2.3)	2012	8 (100)	N	P[b]	0.29% (1/341)	0.29% (1*/341)
	H. lusitanicum	8 (2.3)	2012	8 (100)	N	P[c]	0.29% (1/341)	-
	I. ricinus	60 (17.6)	2011	3 (5)	N	N	-	-
			2012	15 (25)	N	N	-	-
			2013	42 (70)	N	N	-	-
	R. pusillus	1 (0.3)	2013	1 (100)	N	N	-	-
	R. sanguineus	29 (8.5)	2012	23 (79.3)	N	N	-	-
			2013	6 (20.7)	N	N	-	-
Dog	*D. reticulatus*	8 (2.3)	2014	8 (100)	N	P[a]	1.17% (4/341)	-
Deer	*I. ricinus*	98 (28.7)	2015	98 (100)	N	N	-	-
Vegetation	*I. ricinus*	108 (31.7)	2013	108 (100)	N	N	-	-
Total		341 (100)		341			2.3% (8/341)	0.29% (1*/341)

[a] Accession numbers **MF497789-94**: 100% similarity in tul4 gene partial sequence (200 bp) with *Francisella*-like endosymbiont of *Dermacentor reticulatus* (FDrH) strain (EU126640);

[b] Accession number **MF497787**: 98,7% similarity in tul4 gene partial sequence (150 bp) with *Francisella*-like endosymbiont of *Hyalomma marginatum* FLE011 (HQ705174) strain;

[c] Accession number **MF497787**: 97.2% similarity in a 100-bp long fragment of tul4 gene with *Francisella*-like endosymbiont of *Hyalomma aegyptum* (FLE031) strain (HQ705175) and 96.3% similarity in tul4 gene partial sequence (with *Francisella*-like endosymbiont of *Hyalomma marginatum* FLE011 (HQ705174) strain;

4. References

Ahantarig, A., Trinachartvanit, W., Baimai, V., Grubhoffer, L., 2013. Hard ticks and their bacterial endosymbionts (or would be pathogens). Folia Microbiol. (Praha). 58, 419–28. doi:10.1007/s12223-013-0222-1

Baldridge, G.D., Scoles, G.A., Burkhardt, N.Y., Schloeder, B., Kurtti, T.J., Munderloh, U.G., 2009. Transovarial transmission of Francisella-like endosymbionts and Anaplasma phagocytophilum variants in Dermacentor albipictus (Acari: Ixodidae). J. Med. Entomol. 46, 625–32.

Barns, S.M., Grow, C.C., Okinaka, R.T., Keim, P., Kuske, C.R., 2005. Detection of diverse new Francisella-like bacteria in environmental samples. Appl. Environ. Microbiol. 71, 5494–500. doi:10.1128/AEM.71.9.5494-5500.2005

Beard, C.B., Durvasula, R. V, Richards, F.F.,. Bacterial symbiosis in arthropods and the control of disease transmission. Emerg. Infect. Dis. 4, 581–91. doi:10.3201/eid0404.980408

Beati, L., Meskini, M., Thiers, B., Raoult, D., 1997. Rickettsia aeschlimannii sp. nov., a new spotted fever group rickettsia associated with Hyalomma marginatum ticks. Int. J. Syst. Bacteriol. 47, 548–54. doi:10.1099/00207713-47-2-548

Dale, C., Moran, N.A., 2006. Molecular Interactions between Bacterial Symbionts and Their Hosts. Cell 126, 453–465. doi:10.1016/j.cell.2006.07.014

de Carvalho, I.L., Santos, N., Soares, T., Zé-Zé, L., Núncio, M.S., 2011. Francisella-like endosymbiont in Dermacentor reticulatus collected in Portugal. Vector Borne Zoonotic Dis. 11, 185–8. doi:10.1089/vbz.2010.0014

Dennis, D.T., Inglesby, T. V., Henderson, D.A., Bartlett, J.G., Ascher, M.S., Eitzen, E., Fine, A.D., Friedlander, A.M., Hauer, J., Layton, M., Lillibridge, S.R., McDade, J.E., Osterholm, M.T., O'Toole, T., Parker, G., Perl, T.M., Russell, P.K., Tonat, K., 2001. Tularemia as a Biological Weapon. JAMA 285, 2763. doi:10.1001/jama.285.21.2763

Dergousoff, S.J., Chilton, N.B., 2012. Association of different genetic types of Francisella-like organisms with the rocky mountain wood tick (Dermacentor andersoni) and the American dog tick (Dermacentor variabilis) in localities near their northern distributional limits. Appl. Environ. Microbiol. 78, 965–71. doi:10.1128/AEM.05762-11

De Sousa, R., Barata, C., Vitorino, L., Santos-Silva, M., Carrapato, C., Torgal, J., Walker, D., Bacellar, F., 2006. Rickettsia sibirica isolation from a patient and detection in ticks, Portugal. Emerg. Infec. Dis. 12, 1103-1108

Duron, O., 2014. Arsenophonus insect symbionts are commonly infected with APSE, a bacteriophage involved in protective symbiosis. FEMS Microbiol. Ecol. 90, 184–94. doi:10.1111/1574-6941.12381

Duron, O., Noël, V., McCoy, K.D., Bonazzi, M., Sidi-Boumedine, K., Morel, O., Vavre, F., Zenner, L., Jourdain, E., Durand, P., Arnathau, C., Renaud, F., Trape, J.-F., Biguezoton, A.S., Cremaschi, J.,

Dietrich, M., Léger, E., Appelgren, A., Dupraz, M., Gómez-Díaz, E., Diatta, G., Dayo, G.-K., Adakal, H., Zoungrana, S., Vial, L., Chevillon, C., 2015. The Recent Evolution of a Maternally-Inherited Endosymbiont of Ticks Led to the Emergence of the Q Fever Pathogen, Coxiella burnetii. PLoS Pathog. 11, e1004892. doi:10.1371/journal.ppat.1004892

Escudero, R., Toledo, A., Gil, H., Kovácsová, K., Rodríguez-Vargas, M., Jado, I., García-Amil, C., Lobo, B., Bhide, M., Anda, P., 2008. Molecular method for discrimination between Francisella tularensis and Francisella-like endosymbionts. J. Clin. Microbiol. 46, 3139–43. doi:10.1128/JCM.00275-08

Gerhart, J.G., Moses, A.S., Raghavan, R., 2016. A Francisella-like endosymbiont in the Gulf Coast tick evolved from a mammalian pathogen. Sci. Rep. 6, 33670. doi:10.1038/srep33670

Goethert, H.K., Telford, S.R., 2005. A new Francisella (Beggiatiales: Francisellaceae) inquiline within Dermacentor variabilis say (Acari: Ixodidae). J. Med. Entomol. 42, 502–5

Gyuranecz, M., Szeredi, L., Makrai, L., Fodor, L., Mészáros, A.R., Szépe, B., Füleki, M., Erdélyi, K., 2010. Tularemia of European Brown Hare (Lepus europaeus): a pathological, histopathological, and immunohistochemical study. Vet. Pathol. 47, 958–63. doi:10.1177/0300985810369902

Ivanov, I.N., Mitkova, N., Reye, A.L., Hübschen, J.M., Vatcheva-Dobrevska, R.S., Dobreva, E.G., Kantardjiev, T. V, Muller, C.P., 2011. Detection of new Francisella-like tick endosymbionts in Hyalomma spp. and Rhipicephalus spp. (Acari: Ixodidae) from Bulgaria. Appl. Environ. Microbiol. 77, 5562–5. doi:10.1128/AEM.02934-10

Johansson, A., Celli, J., Conlan, W., Elkins, K.L., Forsman, M., Keim, P.S., Larsson, P., Manoil, C., Nano, F.E., Petersen, J.M., Sjöstedt, A., 2010. Objections to the transfer of Francisella novicida to the subspecies rank of Francisella tularensis. Int. J. Syst. Evol. Microbiol. 60, 1717–8; author reply 1718–20. doi:10.1099/ijs.0.022830-0

Karhukorpi, E.K., Karhukorpi, J., 2001. Rapid laboratory diagnosis of ulceroglandular tularemia with polymerase chain reaction. Scand. J. Infect. Dis. 33, 383–5.

Kilpatrick, A.M., Randolph, S.E., 2012. Drivers, dynamics, and control of emerging vector-borne zoonotic diseases. Lancet (London, England) 380, 1946–55. doi:10.1016/S0140-6736(12)61151-9

Kingry, L.C., Petersen, J.M., 2014. Comparative review of Francisella tularensis and Francisella novicida. Front. Cell. Infect. Microbiol. 4, 35. doi:10.3389/fcimb.2014.00035

Kreizinger, Z., Hornok, S., Dán, A., Hresko, S., Makrai, L., Magyar, T., Bhide, M., Erdélyi, K., Hofmann-Lehmann, R., Gyuranecz, M., 2013. Prevalence of Francisella tularensis and Francisella-like endosymbionts in the tick population of Hungary and the genetic variability of Francisella-like agents. Vector Borne Zoonotic Dis. 13, 160–3. doi:10.1089/vbz.2012.1065

Kugeler, K.J., Gurfield, N., Creek, J.G., Mahoney, K.S., Versage, J.L., Petersen, J.M., 2005. Discrimination between Francisella tularensis and Francisella-like endosymbionts when screening ticks by PCR. Appl. Environ. Microbiol. 71, 7594–7. doi:10.1128/AEM.71.11.7594-7597.2005

Liu, J.-N., Yu, Z.-J., Liu, L.-M., Li, N.-X., Wang, R.-R., Zhang, C.-M., Liu, J.-Z., 2016. Identification, Distribution and Population Dynamics of Francisella-like Endosymbiont in Haemaphysalis doenitzi (Acari: Ixodidae). Sci. Rep. 6, 35178. doi:10.1038/srep35178

Liu, L., Li, L., Liu, J., Hu, Y., Liu, Z., Guo, L., Liu, J., 2013. Coinfection of Dermacentor silvarum olenev (acari: ixodidae) by Coxiella-Like, Arsenophonus-like, and Rickettsia-like symbionts. Appl. Environ. Microbiol. 79, 2450–4. doi:10.1128/AEM.03575-12

Lopes de Carvalho, I., Toledo, A., Carvalho, C.L., Barandika, J.F., Respicio-Kingry, L.B., Garcia-Amil, C., García-Pérez, A.L., Olmeda, A.S., Zé-Zé, L., Petersen, J.M., Anda, P., Núncio, M.S., Escudero, R., 2016. Francisella species in ticks and animals, Iberian Peninsula. Ticks Tick. Borne. Dis. 7, 159–65. doi:10.1016/j.ttbdis.2015.10.009

Mörner, T., 1992. The ecology of tularaemia. Rev. Sci. Tech. 11, 1123–30.

Moutailler, S., Valiente Moro, C., Vaumourin, E., Michelet, L., Tran, F.H., Devillers, E., Cosson, J.F., Gasqui, P., Van, V.T., Mavingui, P., Vourc'h, G., Vayssier-Taussat, M., 2016. Co-infection of Ticks: The Rule Rather Than the Exception. PLoS Negl. Trop. Dis. 10, e0004539. doi:10.1371/journal.pntd.0004539

Niebylski, M.L., Peacock, M.G., Fischer, E.R., Porcella, S.F., Schwan, T.G., 1997. Characterization of an endosymbiont infecting wood ticks, Dermacentor andersoni, as a member of the genus Francisella. Appl. Environ. Microbiol. 63, 3933–40.

Noda, H., Munderloh, U.G., Kurtti, T.J., 1997. Endosymbionts of ticks and their relationship to Wolbachia spp. and tick-borne pathogens of humans and animals. Appl. Environ. Microbiol. 63, 3926–32.

Nikoh, N., Hosokawa, T., Moriyama, M., Oshima, K., Hattori, M., Fukatsu, T., 2014. Evolutionary origin of insect-Wolbachia nutritional mutualism. Proc. Natl. Acad. Sci. U. S. A. 111, 10257–62. doi:10.1073/pnas.1409284111

Page, R.D.M., 2003. Tangled Trees: Phylogeny, Cospeciation, and Coevolution.

Petersen, J.M., Mead, P.S., Schriefer, M.E.,. Francisella tularensis: an arthropod-borne pathogen. Vet. Res. 40, 7. doi:10.1051/vetres:2008045

Petersen, J.M., Schriefer, M.E.,. Tularemia: emergence/re-emergence. Vet. Res. 36, 455–67. doi:10.1051/vetres:2005006

Perlman, S.J., Hunter, M.S., Zchori-Fein, E., 2006. The emerging diversity of Rickettsia. Proceedings. Biol. Sci. 273, 2097–106. doi:10.1098/rspb.2006.3541

Potkonjak, A., Gutiérrez, R., Savić, S., Vračar, V., Nachum-Biala, Y., Jurišić, A., Kleinerman, G., Rojas, A., Petrović, A., Baneth, G., Harrus, S., 2016. Molecular detection of emerging tick-borne pathogens in Vojvodina, Serbia. Ticks Tick. Borne. Dis. 7, 199–203. doi:10.1016/j.ttbdis.2015.10.007

Raileanu, C., Moutailler, S., Pavel, I., Porea, D., Mihalca, A.D., Savuta, G., Vayssier-Taussat, M., 2017.

Borrelia Diversity and Co-infection with Other Tick Borne Pathogens in Ticks. Front. Cell. Infect. Microbiol. 7, 36. doi:10.3389/fcimb.2017.00036

Raoult, D., Fournier, P.-E., Abboud, P., Caron, F., 2002. First documented human Rickettsia aeschlimannii infection. Emerg. Infect. Dis. 8, 748–9. doi:10.3201/eid0807.010480

Ryder, J.J., Hoare, M.-J., Pastok, D., Bottery, M., Boots, M., Fenton, A., Atkinson, D., Knell, R.J., Hurst, G.D.D., 2014. Disease epidemiology in arthropods is altered by the presence of nonprotective symbionts. Am. Nat. 183, E89–104. doi:10.1086/674827

Savić, S., Vidić, B., Grgić, Z., Potkonjak, A., Spasojevic, L., 2014. Emerging Vector-Borne Diseases - Incidence through Vectors. Front. public Heal. 2, 267. doi:10.3389/fpubh.2014.00267

Schouls, L., Van de Pol, I., Rijpkema, S., Schot, C.S. 1999. Detection and identification of Ehrlichia, Borrelia burgdorferi sensu lato, and Bartonella species in Dutch Ixodes ricinus ticks. J. Clin. Microbiol. 37, 2215-2222

Scoles, G.A., 2004. Phylogenetic analysis of the Francisella-like endosymbionts of Dermacentor ticks. J. Med. Entomol. 41, 277–86

Sréter-Lancz, Z., Széll, Z., Sréter, T., Márialigeti, K., 2009. Detection of a novel Francisella in Dermacentor reticulatus: a need for careful evaluation of PCR-based identification of Francisella tularensis in Eurasian ticks. Vector Borne Zoonotic Dis. 9, 123–6. doi:10.1089/vbz.2008.0010

Suitor, E.C., Weiss, E., 1961. Isolation af a Rickettsialike Microorganism (Wolbachia Persica, N. SP.) From Argas Persicus (Oken). J. Infect. Dis. 108, 95–106. doi:10.1093/infdis/108.1.95

Tomanović, S., Chochlakis, D., Radulović, Z., Milutinović, M., Cakić, S., Mihaljica, D., Tselentis, Y., Psaroulaki, A., 2013. Analysis of pathogen co-occurrence in host-seeking adult hard ticks from Serbia. Exp. Appl. Acarol. 59, 367–76. doi:10.1007/s10493-012-9597-y

Versage, J.L., Severin, D.D.M., Chu, M.C., Petersen, J.M., 2003. Development of a multitarget real-time TaqMan PCR assay for enhanced detection of Francisella tularensis in complex specimens. J. Clin. Microbiol. 41, 5492–9

Vogler, A.J., Birdsell, D., Price, L.B., Bowers, J.R., Beckstrom-Sternberg, S.M., Auerbach, R.K., Beckstrom-Sternberg, J.S., Johansson, A., Clare, A., Buchhagen, J.L., Petersen, J.M., Pearson, T., Vaissaire, J., Dempsey, M.P., Foxall, P., Engelthaler, D.M., Wagner, D.M., Keim, P., 2009. Phylogeography of Francisella tularensis: global expansion of a highly fit clone. J. Bacteriol. 191, 2474–84. doi:10.1128/JB.01786-08

Weinert, L.A., Werren, J.H., Aebi, A., Stone, G.N., Jiggins, F.M., 2009. Evolution and diversity of Rickettsia bacteria. BMC Biol. 7, 6. doi:10.1186/1741-7007-7-6

Weller, S.J., Baldridge, G.D., Munderloh, U.G., Noda, H., Simser, J., Kurtti, T.J., 1998. Phylogenetic placement of rickettsiae from the ticks Amblyomma americanum and Ixodes scapularis. J. Clin. Microbiol. 36, 1305–17.

3. General Discussion

This thesis aimed the study of host-pathogen interaction systems involving the European wild rabbit (*Oryctolagus cuniculus*) and the Iberian hare (*Lepus granatensis*) with a lagovirus and a zoonotic bacterium. Although the two systems chosen have very different characteristics, their importance is equally recognized, the first by its devastating effects on the affected wild rabbit populations, predatory species and collateral food webs, and the second by its zoonotic potential and consequent Public Health risk.

Amongst the objectives of this study was the investigation of the rabbit haemorrhagic disease virus 2 (RHDV2), a recently emerged and transboundary virus, widely disseminated on the continent and islands which causes a highly contagious haemorrhagic fever in rabbits, usually of fatal outcome. Aspects related to pathology, diagnosis, genetics and epidemiology were addressed.

In more detail, at the beginning of this project we attempted the isolation of RHDV2 in VERO and RK-13 cell lines to ascertain whether this virus differed from classical RHDV, non-cultivable in cell lines **(Study 1)**. The motivation for this assessment was based on the premise that this lagovirus, although related to RHDV, constitutes a genetically different virus (Le Gall-Reculé et al., 2013). Rabbit lagoviruses' non-cultivable nature is largely known as attempts to adapt EBHSV (Gavier-Widén et al., 1991), RHDV (Parra and Prieto, 1990)(Huang et al., 1991) and RCV (Capucci et al., 1996) to grow in various cell lines have repeatedly failed. The inability to grow *Lagovirus* and most caliciviruses *in vitro* is suggested to be associated with virus entry and receptor binding (Guix et al., 2007)(Vashist et al., 2009). This was further demonstrated by transfection studies in which a full-length cDNA clone of RHDV led to the synthesis of infectious virus (Liu, 2006). RHDV was the first calicivirus shown to bind to histo-blood group antigens (HBGAs) of the upper respiratory and/or digestive tract epithelial cells (Ruvoën-Clouet et al., 2000). However, HBGAs are attachment factors (ligands) that facilitate RHDV infection rather than the main cellular receptor (Nyström et al., 2011). The liver is considered the major organ of RHDV replication, but rabbit hepatocytes are completely devoid of HBGAs (Gorvel et al., 1985)(Nyström et al., 2011) which further renders unlikely the use of HBGAs as receptors on hepatocytes and suggests additional mechanisms in the RHDV infection (Nyström et al., 2011).

There is still limited information on the RHDV2 interactions with HBGAs. Nevertheless, our results demonstrated that, after 12 passages, RHDV2 was not adaptable to grow in vitro in the two tested cell lines (RK13 and VERO cells).

Also, at the beginning of our work, we developed and validated of a sensitive and specific molecular method for the detection of RHDV2 (**Study 2**), until then non-existent. Different

molecular assays for the detection of classical RHDV have been described since the late 90s, including conventional RT-PCR assays (Guittré et al., 1995)(Ros Bascunana et al., 1997)(Tham et al., 1999)(Yang et al., 2008), immunocapture-RT-PCR (Le Gall-Reculé, 2001), real time multiplex RT-PCR (Gall et al., 2007), TaqMan real-time RT-PCR (Fitzner et al., 2011) and more recently, loop-mediated isothermal amplification (LAMP) (Yuan et al., 2013) and SYBR green-based real-time PCR (Niedzwiedzka-Rystwej et al., 2013).

Until year 2015, the OIE Reference Laboratory recommended a single step conventional generalist RT-PCR (which primers are able to amplify all RHDV genogroups and RHDV2) followed by sequencing analysis for RHDV2 detection. The method designed by us is currently listed in the OIE Terrestrial Manual, Chapter 2.6.2 Rabbit haemorrhagic disease, where it figures as the recommended real-time RT-qPCR method for the molecular diagnosis of RHDV2.

The interpretation of RT-qPCR results obtained with the method developed during this study, when applied to non-vaccinated animals undergoing acute disease, particularly when low Cq values are obtained, is usually straightforward. In addition, in these clinical forms, histopathological lesions are usually present supporting RHDV2 as the cause of death. Due to the variable mortality rates described for RHDV2 infections (Le Gall-Reculé et al., 2011a)(Dalton et al., 2012)(Le Gall-Reculé et al., 2013), when low viral loads are obtained, differential diagnosis is required since RHDV2 positivity may not necessarily relate to clinical state or fatal outcome. In addition, nucleic acid amplification-based methods have a high sensitivity and low amounts of RHDV2 vaccine virus in tissues of vaccinated animals can influence, or even compromise, the interpretation of results and the final diagnosis. For blue tongue virus (BTV), for example, it was demonstrated that vaccine viral RNA can reach the blood circulation and the spleen in sufficient amounts to be detected by real-time RT-PCR (De Leeuw et al., 2015). Despite previous studies on the RHDV genome persistence in vaccinated and experimentally infected rabbits demonstrated that inactivated vaccine RNA was not detected by RT-qPCR (Gall and Schirrmeier, 2006), the same researchers also showed that genomic RNA or RNA fragments from the closely related RHDV are known to persist for at least 15 weeks after experimental infection (Gall and Schirrmeier, 2006)(Gall et al., 2007). With regards to the challenges that this RHDV2 molecular diagnosis faces in vaccinated animals, we have explored the significance of viral loads in different groups of animals varying in age, immunization status and period between immunization and death, in an opportunist study in a rabbit industry (**Study 3**). We showed that commercial RHDV2 vaccines, even if applied at least 15 days before sampling, do not interfere with the results of the molecular diagnosis by the method that we developed. This study

also demonstrated the interference of maternal antibodies in the efficacy of vaccination in juvenile rabbits, a phenomenon that should be considered when defining vaccination protocols. Humoral immunity is critical to protect rabbits from RHD (Argüello-Villares, 1991)(Huang et al., 1991)(McIntosh et al., 2007) and maternal IgG antibodies, acquired during late pregnancy through the placenta and, later on, via colostrum (Lorenzo Fraile, *personal communication*), may be relevant for young rabbits' resistance to RHDV (Cooke, 2002).

The rapid transmission of RHDV2 from wild to domestic rabbit populations, particularly to traditional rabbitries, often located near rural plots and whose rabbits are often fed with grass harvested in the surrounding fields, was evident in a small study (**Study 4**) reporting the presence of RHDV2 in domestic rabbits from the Azores, a few months after its detection in wild rabbits. The molecular characterization of these viruses revealed that they were identical to the viruses that circulated at the same time in wild rabbits, suggesting transmission to the domestic populations. In nature, the faecal-oral is probably the most important route of infection (Morisse et al., 1991)(Ohlinger et al., 1993), although disease can be originated by oral, nasal or parenteral (intramuscular, intradermal) routes (Argüello-Villares et al., 1988)(Ohlinger et al., 1993). Faeces from surviving rabbits can be infectious for susceptible animals up to 4 weeks after infection (Gregg et al., 1991)(Ohlinger et al., 1991). In Europe, the initial RHDV spread to wild rabbit populations was closely related with the transmission among commercial rabbitries (Cooke et al., 2002). Waste disposal from rabbitries and fresh cut herbage (green feed) used to feed domestic rabbits provided routes for RHDV spread in both directions between wild and captive rabbits (Ohlinger et al., 1994)(Cooke et al., 2002). In this case, the molecular, epidemiological, geographical and ecological data pointed to the dissemination of the virus from the wild to this small backyard premises namely through green feed that rabbit owners collected in the nearby fields.

Given the resistance and easiness of virus transmission, the successful control the disease, and of potential epidemics depends on rapid access to information at the national level on the disease situation (Vallat et al., 2013). Diseases in general, must be immediately notified in a clear and transparent way to ensure a timely response (Vallat et al., 2013). As the OIE official notifications are sporadic, given the urgency in release information to the scientific community as well as to various layers of the society, including conservationists, veterinaries, hunters and hunting tourism agents, reports are often communicated in oral communications and in the format of letters to the editor. This was the case of this short report where we informed on the RHDV2 detection in domestic

rabbits on the Azorean archipelago, only a few months after the disease was first reported in the wild rabbit populations.

In **Study 5** we evaluated the effectiveness of vaccination for RHDV2 as a therapeutic tool at the population level. Given that immunoglobulins are detected as early as 7 to 10 days after immunization, vaccination can be used as an effective post-exposure treatment on farms with separate sheds and good biosecurity measures (OIE Terrestrial Manual, 2016).

Although vaccination proved effective in controlling the disease in a post-infection scenario, the long-time span between the introduction of vaccination and the drop of mortality rates clearly reinforces the importance of implementing vaccination as a preventive measure whenever possible.

In **Study 6**, essentially epidemiological, we investigated a wild rabbit breeding unit located in Barrancos, Portugal, where mortality due to the haemorrhagic viral disease had recently occurred. The animals were intended to be released in areas designated for the reintroduction of the endangered Iberian lynx (*Lynx pardinus*), also reproduced in captivity. The results of this investigation showed a close genetic relationship between the strains identified in this 2016' outbreak and strains that had circulated previously in that geographical region, the first being detected in the same unit in 2012. These results suggest the existence of mechanisms that allow the maintenance of the virus in nature, and the possible role of other species as virus reservoirs. For RHDV, besides direct transmission through rabbit contact with diseased rabbits or rabbit carcasses, indirect transmission by mechanical vectors such as insects (Asgari et al., 1998)(McColl et al., 2002a) and seabirds (Cooke et al., 2002) was recognised. In addition, some micromammals species are reservoirs and potential sources of RHDV (Merchán et al., 2011) and there are serological evidences of the capability of RHDV to infect a wider broad of hosts, in red foxes and scavengers living in sympathy with RHDV infected rabbit populations (Frolich et al., 1998)(Leigthon et al., 1995)(Parkes et al., 2004). This data may imply a relevant role of other species in the epidemiology and persistence of the disease (Merchán et al., 2011). Despite sympatric species were not sampled and tested in this study, the results reinforced the notion that RHDV2 will continue to pose major challenges to the recovery of leporids populations in the country, with the consequent deleterious impact on the species that depend on it, as well as, on collateral food webs where the rabbit predators find alternatives for survival.

Despite the dramatic reduction of the populations in certain areas of the national territory, the wild rabbits accumulate paradoxical labels in the Iberia, being considered a pest in a few locations where its presence is detrimental (Ferreira, 2012)(Ferreira and Delibes-Mateos, 2010). The Berlengas

archipelago is a rich and unique terrestrial ecosystem (Catry et al., 2010), where the wild rabbit has had a disturbing role in its original ecology (Amado et al., 2007). The success of alien species eradication from islands was considered a major achievement in conservation biology (Courchamp et al., 2003). While management of this invasive species in the insular ecosystem of Berlengas is currently being carried out, we made a last effort to identify the causes of mortality on the rabbits in the Berlenga Island. We detected and characterized RHDV2 strain putatively representative of the last passage of the virus through this small archipelago (**Study 7**). In view of the currently known strains, the results obtained in this study suggested that the virus may have been introduced into the island some years ago and, due to insularity, may have evolved separately from the strains circulating in the continent.

The genetic variability and evolution of RHDV2 in Portugal were investigated in two phylogenetic studies also included in this thesis, based on the *vp60 gene* characterization of RHDV2 strains obtained in Portugal between 2012 and 2017.

The first study (**Study 8**) aimed to characterize the RHDV2 strains circulating in the Azorean archipelago where the virus was first detected by the end of 2014. Nucleotide analysis showed that the strains from Azores are closely related to each other, sharing a high genetic identity (>99.15%) and forming a cluster, separately from the strains originating in Europe. Bayesian and maximum likelihood (ML) phylogenetic analyses disclosed that strains from Azores were more closely to a few strains from South of mainland Portugal than with any other known at that time. In the genomic region analysed, comprehending the terminal 942 nucleotides of the *vp60* gene, four new single nucleotide polymorphisms (SNP) were identified. This genetic signature may constitute putative geographic molecular markers for the RHDV2 Azorean strains if persisting in the future.

In **Study 9**, we characterized seven RHDV2 strains obtained during the first outbreak of the disease in the Madeira archipelago. The results evidenced that these viruses also have unique genetic markers in the *vp60* gene. Two are expressed at the protein level involving positions 480 and 570 of the 579 amino acid-long protein. Though the Asn_{480} or Ser_{480} and Glu_{570} residues identified in the strains from Madeira are located in loops of the VP60 protein, Asn_{480} potentially impacts on the secondary structure of the capsid protein, resulting in the replacement of an α-helix by a longer β-strand. Along with these, we identified eight additional SNPs that were frequently found in the strains from Madeira but were rarely detected in strains from other geographic regions. Altogether, the SNPs defined a "DNA fingerprint" for the viruses that circulate in this archipelago. Through this investigation, we could trace back the origin of the Madeira archipelago outbreak to mainland

Portugal (604 miles away) and exclude the hypothesis of spreading from the nearer Canary Islands (324 miles away), where RHDV2 had been detected before. The conclusions emphasize the fact that RHDV2 dissemination depends on other factors rather than geographical proximity, also suggested for the RHDV2 introduction in the Azorean archipelago (**Study 8**).

As a common trace for these eight studies, we extend the knowledge on the genetic and epidemiology of RHDV2 after developing, validating and testing a RHDV2 method in non-vaccinated and vaccinated animals. During these years, we had the opportunity of investigating outbreaks in the industry and in the wild and exploring the phylogenetic relationships among strains originated in Europe and traced probable dissemination pathways between geographic distinct areas. By publishing our results we stimulated, at the international level, several areas of research concerning the pathogen itself and dissemination factors of the disease. We raised awareness for the veterinarians and other animal health professionals and we provided considerations at the ecosystem level and species conservation. Seven years after its first detection, the recovering of the wild populations is far from satisfactory, affecting a gross share of the touristic market associated directly and indirectly with cinegetic activities in the Iberia and leading to illegal movement of animals without genetic and sanitary evaluations. Moreover, recent data has showed that RHDV2 is already affecting consistently the European hare in Europe (Lavazza A., *personal communication, June 2017*).

Within the frame of this thesis, we also aimed to evaluate the interaction between the wild leporids, Iberian hare and wild rabbit, and the bacterium *Francisella tularensis*. Although this bacterial infection can occur asymptomatically in leporids, the Public Health risk is unequivocal considering that *F. tularensis* is a zoonotic bacterium (reviewed in Carvalho et al. (2014)) and the importance of leporids in hunting activity in Portugal (Ferreira and Ferreira, 2014)(Calvete et al., 2004)(Beja et al., 2007), which potentiates animal-human contact.

The Iberian hare (*Lepus granatensis*) is the most abundant of the two hare species endemic in the Iberian Peninsula (Duarte, 2000)(Smith and Boyer, 2008)(Alves et al., 2003), being an alternative prey for the carnivores that depend on the wild rabbit (Acevedo et al., 2012). In addition, it could represent a sentinel species for the infection by *Francisella tularensis* (Duarte, 2000). Although *F. tularensis* subsp. *holarctica* was detected for the first time in Portugal in 2007 (de Carvalho et al., 2007), the systematic screening of this bacterium in wild leporid tissues, considered the main reservoirs of this pathogen (reviewed in Carvalho et al. (2014)), was never performed. Hence, a

systematic approach by molecular methods was conducted to investigate the presence of this agent in samples of wild leporids (**Study 12**) collected from geographical locations where positive human serology had been registered in 1999 (Núncio, 2002). The results confirmed that *F. tularensis* circulates in wild leporids in Portugal, at least in that geographic area, and probably even before 1999. The bacterium was detected in tissue samples of Iberian hares, but also in wild rabbits (*O. cuniculus*) where *F. tularensis* had never been detected previously. Indeed, both the European wild rabbit (*O. cuniculus*) and the domestic rabbit are both relatively resistant to tularaemia (Gyuranecz et al., 2010). Interestingly, in a recently published study on the results of the surveillance of tularaemia in wildlife in France (2002-2003 and 2012-2013 hunting seasons), *F. tularensis* subsp. *holarctica* was also detected in four wild rabbits (*O. cuniculus*) (Moinet et al., 2016).

We also carried out studies on known arthropod vectors of *F. tularensis* that showed ticks as the main vector of this bacterium in our country. Approximately 5000 mosquitoes from five different genus (namely *Culex* spp., *Ochlerotatus* spp., *Anopheles* spp., *Culiseta* spp., and a small number of *Aedes aegypti* females from the Madeira Island) were investigated for the presence of *F. tularensis* all testing negative (**Annex V**). Contrarily, the pathogen was identified in several tick species in Portugal (**Study 12**). Ticks are not only the vector for the transmission of *F. tularensis* but can potentially maintain the organism in nature between epizootics (Ellis et al., 2002). Moreover, the screening of ticks collected from human hosts for the presence of *F. tularensis* allowed the detection of a *Hyalomma marginatum* tick co-infected with both *Francisella*-like endosymbiont (FLE) and *Rickettsia aeschlimannii*, a pathogenic rickettsia belonging to the Spotted-Fever Group (SFG) (**Study 13**). Although the infection of *Hyalomma* ticks with FLEs has been described before, namely in *H. marginatum*, *H. aegyptum* (Ivanov et al., 2011) and *H. lusitanicum* (Lopes de Carvalho et al., 2016), to our knowledge, the co-infection with FLEs and *R. aeschlimannii* is a novelty. *R. aeschlimannii* was isolated for the first time in *H. marginatum* in 1997 (Beati et al., 1997) and was recognized as a pathogenic agent and included in the SFG in 2002 (Raoult et al., 2002). The pathogenicity of FLEs to humans and animals requires further investigation. Overall, endosymbionts appear to be harmless to mammals and there is no evidence of FLEs' pathogenicity to humans (Gerhart et al., 2016). Some FLEs seem to lack or at least have a significantly different RD1 (region of difference) sequence, an important molecular marker for the discrimination of pathogenic *F. tularensis* subspecies (Ivanov et al., 2011). Nevertheless, these bacteria have been detected in small free-living mammals in Europe, highlighting the need to clarify the potential transmission of some

FLE strains from ticks to mammals, if present in the salivary glands or, alternatively, ingested along with ticks via grooming (Lopes de Carvalho et al., 2016)

Finally, the isolation of this fastidious bacterium has been attempted by the classical standard methods described in the literature but also by using an innovative technique including an *in vivo* passage (**Study 11**). This technique is carried out in the Institute for Veterinary Medical Research (Centre for Agricultural Research, Hungarian Academy of Sciences), an OIE reference laboratory for *F. tularensis*. Despite metagenomic approches to identify non-cultivable microrganisms, using NGS (Stewart et al., 2012)(Albanese et al., 2015), *F. tularensis* isolation would have allowed its comprehensive characterization and full assessment of its virulence potential (Vartoukian et al., 2010). Despite all efforts, the attempts to isolate *F. tularensis* were unsuccessful.

As a common denominator for these studies, we extend the knowledge on the genetic *of F. tularensis* and ascertain the tularaemia epidemiologic situation in Portugal by confirming leporids as reservoirs and ticks as the main vectors for the pathogen, while reinforcing the need to further understand the role of FLE in the transmission of this or other vector-borne organisms.

Finally, during the progression of this work we produced and deposited several nucleotide sequences of RHDV2 (n=80), F. *tularensis* subsp. *holarctica* (n=1) and *Francisella*-like endosymbionts (FLEs) (n=8), in public databases (GenBank). Sharing genomic data among the scientific community is essential for a better perception of the evolution of these microorganisms and especially valuable when associated with epidemiological data. The accession numbers of all sequences produced during this work are disclosed in **Annex VI**.

As a common denominator for these studies, we produced data that will support future studies regarding the two leporid pathogens, in the veterinarian, public health and species conservation perspectives.

To carry out the investigations presented in this thesis, we used classical methodologies of pathology, virology and bacteriology, such as methods of tissues inclusion, cuts and staining, preparation of macerates, bacterial and tissue cultures. The molecular methodologies used included cloning, different PCR formats, sequencing and *in vitro* transcription. With regards to bioinformatics for sequence analysis, we used several methods of phylogenetic analysis (NJ, ML,

Bayesian, Network), for screening of recombination (MaxChi, Phylogenetic profiling, Slidingwindow phylogeny), and temporal evolution (Tempest), among others.

4. Future prespectives

Molecular epidemiology is a powerful tool to trace the routes of viral dissemination and to understand viral evolution. However, the inference depicted by these analyses is dependent on the representativeness of sequences publically available at the time. Retrospective studies help to reframe these analyses, providing more realistic epidemiological snapshots and refining the evolution history of the viruses.

Within the time frame of this work, we gathered an extensive database of complete RHDV2 *vp60* sequences (n=80) of which a fraction (n=33) had already been made publically available. Forty-seven new RHDV2 sequences obtained from samples collected between 2012 and 2016 are currently under study. Preliminary results show that these sequences will bring new insights on the phylogenetic relationships among RHDV2 strains that have been circulating in Portugal since 2012. This is particularly true for the 15 sequences originated from the North of Portugal, since only two sequences from this geographic region are currently publically available. Despite ongoing, the preliminary but promising analysis provides a glimpse on the population dynamics of RHDV2 on the mainland and refines the evolution history of RHDV2 in Europe. The results not only will allow to reinforce the notion of effective geographic segregation of insular strains (Azores and Madeira archipelagos), but will also show that the pattern of virus dispersion at continental level is still relatively preserved in the North (where most of the sequences are monophyletic), in contrast with a more evident mixture between strains in the Centre and South. In addition, our data seems to confirme that, regarding the evolution history of RHDV2 in Europe, the southern European strains (Spain, Portugal, Malta) have a closer genetic proximity of with sequences from Sweden, rather than with those from France and Italy. This is a line of investigation that is currently being followed and will proceed in the future.

Recently information on the wild rabbit populations from two Azorean islands described a high RHDV2 seroprevalence (40% and 70%, respectively) on wild rabbits. Despite these findings may reflect a favourable evolution of the host-virus equilibrium in these areas, additional and continued investigation on the immunological resistance of these populations is necessary to understand the impact of herd immunity in the population’s recovery. The high evolution-rate of RHDV2 and the possibility of new introductions with different strains, pose a continuous threat to the susceptible populations. Furthermore, RHDV2 was shown to affect Iberian hares (Lavazza A, *personal communication*), showing that this virus is no longer considered a sole host specific virus and has now widen its host range to better survive and persist. It is yet unpredictable whether virus-host

equilibrium will be ever attained and it is still unknown what impact will RHDV2 have in the Iberian hare populations, currently considered stable. This line of investigation is particular interesting for the future.

Currently, the wild rabbit fragile situation in our country is still alarming, leading the Portuguese Government to activate a plan aiming the control of the disease (Despatch 4757/2017 of May 31st).

In this critical scenario, the development of a disease control tool, namely a safe RHDV2 recombinant vaccine will be essential towards the control of the virus in the field and constitutes another line of investigation that we would like to follow in a nearby future.

Finally, our phylogenetic analyses, based on the *vp60* capsid gene, have not evidenced recombination events. However, other researchers have identified potential points of recombination between the non-structural and structural encoding genes and more recently near the 5' end of the genome. In the future, we would like to explore the role of the recombination events on the evolution and pathogenesis of RHDV2.

Regarding *Francisella tularensis*, despite the bacterium was detected during this study in wild leporids, the full characterization of the strains is still missing and would provide important information on the *Francisella tularensis* subsp. *holarctica* strains that circulate in Portugal. In a nearby future, we intend to complete the molecular characterization namely resourcing to metagenomic by next-generation sequencing methods.

The true animal reservoirs of *F. tularensis* in different geographical areas of Portugal must be better defined. Further characterisation of wild animal species that can carry *F. tularensis* for extended periods in their natural habitat is needed.

From the public health point of view, the potential impact of tularaemia as an emerging zoonosis should not be neglected. Moreover, surveillance of this disease in sentinel animals is essential for the monitoring and prevention of eventual epidemic outbreaks, especially in regions where contact with potential reservoirs and vectors are more frequent.

The reservoir competence of arthropods, especially ticks, for *F. tularensis* subsp. *holarctica* must be further assessed, once different tick species were detected infected.

5. References

Abrantes, J., Lopes, A.M., Dalton, K.P., Parra, F., Esteves, P.J., 2014. Detection of RHDVa on the Iberian Peninsula: Isolation of an RHDVa strain from a Spanish rabbitry. Arch. Virol. 159, 321–326. doi:10.1007/s00705-013-1808-2

Abrantes, J., Lopes, A.M., Dalton, K.P., Melo, P., Correia, J.J., Ramada, M., Alves, P.C., Parra, F., Esteves, P.J., 2013. New variant of rabbit hemorrhagic disease virus, Portugal, 2012-2013. Emerg. Infect. Dis. 19, 1900–2. doi:10.3201/eid1911.130908

Abrantes, J., van der Loo, W., Le Pendu, J., Esteves, P.J., 2012. Rabbit haemorrhagic disease (RHD) and rabbit haemorrhagic disease virus (RHDV): a review. Vet. Res. doi:10.1186/1297-9716-43-12

Abrantes, J., Esteves, P.J., 2010. Not-so-novel michigan rabbit calicivirus. Emerg. Infect. Dis. doi:10.3201/eid1608.091803

Abrantes, J., Esteves, P.J., Van Der Loo, W., 2008. Evidence for recombination in the major capsid gene VP60 of the rabbit haemorrhagic disease virus (RHDV). Arch. Virol. 153, 329–335. doi:10.1007/s00705-007-1084-0

Acevedo P., Melo-Ferreira J., Real R., Alves P.C., 2012. Past, Present and Future Distributions of an Iberian Endemic, Lepus granatensis: Ecological and Evolutionary Clues from Species Distribution Models PLoS One. 7(12): e51529. doi: 10.1371/journal.pone.0051529

Adams, P.D., Afonine, P. V., Bunkóczi, G., Chen, V.B., Davis, I.W., Echols, N., Headd, J.J., Hung, L.-W., Kapral, G.J., Grosse-Kunstleve, R.W., McCoy, A.J., Moriarty, N.W., Oeffner, R., Read, R.J., Richardson, D.C., Richardson, J.S., Terwilliger, T.C., Zwart, P.H., 2010. *PHENIX*: a comprehensive Python-based system for macromolecular structure solution. Acta Crystallogr. Sect. D Biol. Crystallogr. 66, 213–221. doi:10.1107/S0907444909052925

Ahantarig, A., Trinachartvanit, W., Baimai, V., Grubhoffer, L., 2013. Hard ticks and their bacterial endosymbionts (or would be pathogens). Folia Microbiol. (Praha). 58, 419–28. doi:10.1007/s12223-013-0222-1

Ahmad, S.T., El-Samadony, H.A., Mahgoub, K.M., 2011. Immunological and Virological Studies on Rabbit Hemorrhagic Disease Virus. Glob. Vet. 7, 545–556

Albanese, D., De Filippo, C., Cavalieri, D., Donati, C., 2015. Explaining Diversity in Metagenomic Datasets by Phylogenetic-Based Feature Weighting. PLOS Comput. Biol. 11, e1004186. doi:10.1371/journal.pcbi.1004186

Alda, F., Doadrio, I., 2014. Spatial genetic structure across a hybrid zone between European rabbit subspecies. PeerJ. doi:10.7717/peerj.582

Alda, F., Gaitero, T., Suárez, M., Merchán, T., Rocha, G., Doadrio, I., 2010. Evolutionary history and molecular epidemiology of rabbit haemorrhagic disease virus in the Iberian Peninsula and Western Europe. BMC Evol. Biol. 10, 347. doi:10.1186/1471-2148-10-347

Ales, R.F., Martin, A., Ortega, F., Ales, E.E., 1992. Recent changes in landscape structure and function in a mediterranean region of SW Spain (1950–1984). Landsc. Ecol. 7, 3–18. doi:10.1007/BF02573953

Alkhuder, K., Meibom, K.L., Dubail, I., Dupuis, M., Charbit, A., 2009. Glutathione provides a source of cysteine essential for intracellular multiplication of Francisella tularensis. PLoS Pathog. 5, e1000284.

doi:10.1371/journal.ppat.1000284

Almeida, T., Lopes, A.M., Magalhães, M.J., Neves, F., Pinheiro, A., Gonçalves, D., Leitão, M., Esteves, P.J., Abrantes, J., 2015. Tracking the evolution of the G1/RHDVb recombinant strains introduced from the Iberian Peninsula to the Azores islands, Portugal. Infect. Genet. Evol. 34, 307–13. doi:10.1016/j.meegid.2015.07.010

Alonso, C., Oviedo, J.M., Martín-Alonso, J.M., Díaz, E., Boga, J.A., Parra, F., 1998. Programmed cell death in the pathogenesis of rabbit hemorrhagic disease. Arch. Virol. 143, 321–32

Alves, P.C., Ferrand, N., Suchentrunk, F., Harris, D.J., 2003. Ancient introgression of Lepus timidus mtDNA into *L. granatensis* and *L. europaeus* in the Iberian Peninsula. Mol. Phylogenet. Evol. 27, 70–80. doi:10.1016/S1055-7903(02)00417-7

Alves, P.C., Gonçalves, H., Santos, M., Rocha, A., 2002. Original investigation Reproductive biology of the Iberian hare, *Lepus granatensis*, in Portugal. Mamm. Biol. 67, 358–371. doi:10.1078/1616-5047-00051

Alves, P.C., Rocha, A., 2003. Environmental factors have little influence on the reproductive activity of the Iberian hare (Lepus granatensis). Wildl. Res. 30, 639–647. doi:10.1071/WR02035

Amado, A., Gafeira, C., Teixeira, A., Preto, A., 2007. Relatório do Plano de Ordenamento da Reserva Natural das Berlengas. Instituto de Conservação da Natureza e Florestas, I.P. (*in Portuguese*)

Anand, N., Deochand, O., Murphy, R., 2017. Imaging Findings of Ulceroglandular Tularemia. J. Radiol. Case Rep. 11, 1–6. doi:10.3941/jrcr.v11i1.2983

Angerbjörn A., Flux J.E.C., 1995. *Lepus timidus*. Mammalian Species, 495, 1–11

Angerman R., 1974. Les Lagomorphes. In : Grzimek, Le Monde Animal, XII, Mammifères 3, Stauffacher ed : 373-411

Antwerpen, M.H., Prior, K., Mellmann, A., Höppner, S., Splettstoesser, W.D., Harmsen, D., 2015. Rapid high resolution genotyping of Francisella tularensis by whole genome sequence comparison of annotated genes ("MLST+"). PLoS One 10, e0123298. doi:10.1371/journal.pone.0123298

Aragão, H.B., 1927. Myxoma of rabbits. Mem. Inst. Oswaldo Cruz 20, 237-247

Argüello-Villares, J.L., 1991. Viral haemorrhagic disease of rabbits: vaccination and immune response. Rev. Sci. Tech. 10, 459–80

Argüello-Villares, J., Llanos Pellitero, A., Pérez-Ordoyo Garcia, L., 1988. Enfermedad vírica hemorragica canejo en España. Rev. Med. Vet 5, 645–650

Arnaud J.M., 1987. Os concheiros mesolíticos dos vales do Tejo e Sado: semelhanças e diferenças. Arqueologia15, 53-64

Asgari, S., Hardy, J.R., Cooke, B.D., 1999. Sequence analysis of rabbit haemorrhagic disease virus (RHDV) in Australia: alterations after its release. Arch. Virol. 144, 135–45

Asgari, S., Hardy, J.R.E., Sinclair, R.G., Cooke, B.D., 1998. Field evidence for mechanical transmission of rabbit haemorrhagic disease virus (RHDV) by flies (Diptera: Calliphoridae) among wild rabbits in Australia. Virus Res. 54, 123–132. doi:10.1016/S0168-1702(98)00017-3

Ashtekar, A.R., Katz, J., Xu, Q., Michalek, S.M., 2012. A mucosal subunit vaccine protects against lethal respiratory infection with *Francisella tularensis* LVS. PLoS One 7, e50460

doi:10.1371/journal.pone.0050460

Avise, J.C., Hamrick, J.L., 1996. Conservation genetics: case histories from nature. Conservation Genetics. Chapman&Hall, New York-London

Baily, J.L., Dagleish, M.P., Graham, M., Maley, M., Rocchi, M.S., 2014. RHDV variant 2 presence detected in Scotland. Vet. Rec. 174, 411–411. doi:10.1136/vr.g2781

Balagopal, A., MacFarlane, A.S., Mohapatra, N., Soni, S., Gunn, J.S., Schlesinger, L.S., 2006. Characterization of the receptor-ligand pathways important for entry and survival of *Francisella tularensis* in human macrophages. Infect. Immun. 74, 5114–5125. doi:10.1128/IAI.00795-06

Baldridge, G.D., Scoles, G.A., Burkhardt, N.Y., Schloeder, B., Kurtti, T.J., Munderloh, U.G., 2009. Transovarial transmission of *Francisella*-like endosymbionts and *Anaplasma phagocytophilum* variants in *Dermacentor albipictus* (Acari: Ixodidae). J. Med. Entomol. 46, 625–32

Ballesteros F., 1998. Las especies de caza en España. Biología, ecología y conservación. Ref: L0015168. ISBN 8460582736. Editorial Estudio y Gestión del Medio editado por Estudio y Gestión del Medio

Banada, P.P., Deshpande, S., Chakravorty, S., Russo, R., Occi, J., Meister, G., Jones, K.J., Gelhaus, C.H., Valderas, M.W., Jones, M., Connell, N., Alland, D., 2017. Sensitive Detection of *Francisella tularensis* Directly from Whole Blood by Use of the GeneXpert System. J. Clin. Microbiol. 55, 291–301. doi:10.1128/JCM.01126-16

Bandelt, H.J., Forster, P., Röhl, A., 1999. Median-joining networks for inferring intraspecific phylogenies. Mol. Biol. Evol. 16, 37–48

Bandelt H.J., Forster P., Sykes B.C., Richards M.B., 1995. Mitochondrial portraits of human populations using median networks. Genetics 141(2): 743–747

Bandelt H.J., Dress A.W., 1992. Split decomposition: a new and useful approach to phylogenetic analysis of distance data. Mol Phylogenet Evo 1(3): 242–252

Bandouchova, H., Pohanka, M., Vlckova, K., Damkova, V., Peckova, L., Sedlackova, J., Treml, F., Vitula, F., Pikula, J., 2011. Biochemical responses and oxidative stress in *Francisella tularensis* infection: a European brown hare model. Acta Vet. Scand. 53, 2. doi:10.1186/1751-0147-53-2

Barbieri I., Lavazza A., Brocchi E., Konig M., and Capucci, L., 1997. Morphological, structural and antigenic modifications of rabbit haemorrhagic disease virus in the course of the disease. In: Chasey D., Gaskell R.M., Clarke I.N. (eds) Proceedings of the 1st symposium on calicivirus of the European Society of Veterinary Virology (ESVV), Reading, UK, 182–193

Bárcena, J., Guerra, B., Angulo, I., González, J., Valcárcel, F., Mata, C.P., Castón, J.R., Blanco, E., Alejo, A., 2015. Comparative analysis of rabbit hemorrhagic disease virus (RHDV) and new RHDV2 virus antigenicity, using specific virus-like particles. Vet. Res. 46, 106. doi:10.1186/s13567-015-0245-5

Bárcena, J., Verdaguer, N., Roca, R., Morales, M., Angulo, I., Risco, C., Carrascosa, J.L., Torres, J.M., Castón, J.R., 2004. The coat protein of Rabbit hemorrhagic disease virus contains a molecular switch at the N-terminal region facing the inner surface of the capsid. Virology 322, 118–134. doi:10.1016/j.virol.2004.01.021

Bárcena, J., Morales, M., Vázquez, B., Boga, J.A., Parra, F., Lucientes, J., Pagès-Manté, A., Sánchez-

Vizcaíno, J.M., Blasco, R., Torres, J.M., 2000. Horizontal transmissible protection against myxomatosis and rabbit hemorrhagic disease by using a recombinant myxoma virus. J. Virol. 74, 1114–1123. doi:10.1128/JVI.74.3.1114-1123.2000

Barel, M., Charbit, A., 2017. Role of Glycosylation/Deglycolysation Processes in *Francisella tularensis* Pathogenesis. Front. Cell. Infect. Microbiol. 7, 1–6. doi:10.3389/fcimb.2017.00071

Barel, M., Hovanessian, A.G., Meibom, K., Briand, J.-P., Dupuis, M., Charbit, A., 2008. A novel receptor - ligand pathway for entry of *Francisella tularensis* in monocyte-like THP-1 cells: interaction between surface nucleolin and bacterial elongation factor Tu. BMC Microbiol. 8, 145. doi:10.1186/1471-2180-8-145

Barns, S.M., Grow, C.C., Okinaka, R.T., Keim, P., Kuske, C.R., 2005. Detection of diverse new *Francisella*-like bacteria in environmental samples. Appl. Environ. Microbiol. 71, 5494–500. doi:10.1128/AEM.71.9.5494-5500.2005

Barratt, B.I.P., Ferguson, C.M., Heath, A.C.G., Evans, A.A., Logan, R.A.S., 1998. Can insects transmit rabbit haemorrhagic disease virus?. Proceedings of the 51st New Zealand Plant Protection Conference. 245-250.

Barthelemy J.P., 1989. Fikdm copair hypergraphs to median graphs with latent vertices. Discrete Mathematics 76

Beati, L., Meskini, M., Thiers, B., Raoult, D., 1997. *Rickettsia aeschlimannii* sp. nov., a new spotted fever group rickettsia associated with *Hyalomma marginatum* ticks. Int. J. Syst. Bacteriol. 47, 548–54. doi:10.1099/00207713-47-2-548

Becker, S., Lochau, P., Jacob, D., Heuner, K., Grunow, R., 2016. Successful re-evaluation of broth medium T for growth of *Francisella tularensis* ssp. and other highly pathogenic bacteria. J. Microbiol. Methods 121, 5–7. doi:10.1016/j.mimet.2015.11.018

Beja, P., Pais, M., Palma, L., 2007. Rabbit *Oryctolagus cuniculus* Habitats in Mediterranean Scrubland: The Role of Scrub Structure and Composition. Wildlife Biol. 13, 28–37. doi:10.2981/0909-6396(2007)13[28:ROCHIM]2.0.CO;2

Bennett, G.M., Moran, N.A., 2015. Heritable symbiosis: The advantages and perils of an evolutionary rabbit hole. Proc. Natl. Acad. Sci. U. S. A. 112, 10169–76. doi:10.1073/pnas.1421388112

Bergin, I.L., Wise, A.G., Bolin, S.R., Mullaney, T.P., Kiupel, M., Maes, R.K., 2009. Novel calicivirus identified in rabbits, Michigan, USA. Emerg. Infect. Dis. 15, 1955–62. doi:10.3201/eid1512.090839

Bertagnoli, S., Gelfi, J., Le Gall, G., Boilletot, E., Vautherot, J.F., Rasschaert, D., Laurent, S., Petit, F., Boucraut-Baralon, C., Milon, A., 1996a. Protection against myxomatosis and rabbit viral hemorrhagic disease with recombinant myxoma viruses expressing rabbit hemorrhagic disease virus capsid protein. J. Virol. 70, 5061–6

Bertagnoli, S., Gelfi, J., Petit, F., Vautherot, J.F., Rasschaert, D., Laurent, S., Le Gall, G., Boilletot, E., Chantal, J., Boucraut-Baralon, C., 1996b. Protection of rabbits against rabbit viral haemorrhagic disease with a vaccinia-RHDV recombinant virus. Vaccine 14, 506–10

Best, S.M., Kerr, P.J., 2000. Coevolution of host and virus: the pathogenesis of virulent and attenuated

strains of myxoma virus in resistant and susceptible european rabbits. Virology 267, 36–48. doi:10.1006/viro.1999.0104

Biju-Duval, C., Ennafaa, H., Dennebouy, N., Monnerot, M., Mignotte, F., Soriguer, R.C., Gaa 'ied, A.E., Hill, A.E., Mounolou, J.-C., 1991. Journal of Molecular Evolution Mitochondrial DNA Evolution in Lagomorphs: Origin of Systematic Heteroplasmy and Organization of Diversity in European Rabbits. J Mol Evol 33, 92–102.

Birdsell, D.N., Johansson, A., Öhrman, C., Kaufman, E., Molins, C., Pearson, T., Gyuranecz, M., Naumann, A., Vogler, A.J., Myrtennäs, K., Larsson, P., Forsman, M., Sjödin, A., Gillece, J.D., Schupp, J., Petersen, J.M., Keim, P., Wagner, D.M., 2014. *Francisella tularensis* subsp. *tularensis* Group A.I, United States. Emerg. Infect. Dis. 20, 861–5. doi:10.3201/eid2005.131559

Boga, J.A., Casais, R., Marin, M.S., Martin-Alonso, J.M., Carmenes, R.S., Prieto, M., Parra, F., 1994. Molecular cloning, sequencing and expression in *Escherichia coli* of the capsid protein gene from rabbit haemorrhagic disease virus (Spanish isolate AST/89). J. Gen. Virol. 75, 2409–2413. doi:10.1099/0022-1317-75-9-2409

Branco, M., Ferrand, N., 2002. Genetic polymorphism of antithrombin III, haptoglobin, and haemopexin in wild and domestic European rabbits. Biochem. Genet. Dec; 40(11-12):387-93

Branco, M., Ferrand, N., Monnerot, M., 2000. Phylogeography of the European rabbit (*Oryctolagus cuniculus*) in the Iberian Peninsula inferred from RFLP analysis of the cytochrome b gene. Heredity (Edinb). 85, 307–317. doi:10.1046/j.1365-2540.2000.00756.x

Branco, M., Monnerot, M., Ferrand, N., Templeton, A.R., 2002. Postglacial dispersal of the European rabbit (*Oryctolagus cuniculus*) on the Iberian peninsula reconstructed from nested clade and mismatch analyses of mitochondrial DNA genetic variation. Evolution 56, 792–803

Breimer, M.E., Hansson, G.C., Karlsson, K.A., Leffler, H., Pimlott, W., Samuelsson, B.E., 1979. Selected ion monitoring of glycosphingolipid mixtures. Identification of several blood group type glycolipids in the small intestine of an individual rabbit. Biol. Mass Spectrom. 6, 231–241. doi:10.1002/bms.1200060603

Broekhuijsen, M., Larsson, P., Johansson, A., Byström, M., Eriksson, U., Larsson, E., Prior, R.G., Sjöstedt, A., Titball, R.W., Forsman, M., 2003. Genome-wide DNA microarray analysis of *Francisella tularensis* strains demonstrates extensive genetic conservation within the species but identifies regions that are unique to the highly virulent *F. tularensis* subsp. *tularensis*. J. Clin. Microbiol. 41, 2924–31

Bröms, J.E., Meyer, L., Sjöstedt, A., 2016. A mutagenesis-based approach identifies amino acids in the N-terminal part of *Francisella tularensis* IglE that critically control Type VI system-mediated secretion. Virulence 00–00. doi:10.1080/21505594.2016.1258507

Brotcke, A., Weiss, D.S., Kim, C.C., Chain, P., Malfatti, S., Garcia, E., Monack, D.M., 2006. Identification of MglA-regulated genes reveals novel virulence factors in *Francisella tularensis*. Infect. Immun. 74, 6642–6655. doi:10.1128/IAI.01250-06

Bull R., White P., 2010. Genome organization and recombination, Chapter 3. In: Hansman GS, Jiang XJ, Green KY (eds) Caliciviruses: molecular and cellular virology. Caister Academic Press, ISBN: 978-1-

904455-63-9

Burrows, S. M., Elbert, W., Lawrence, M. G. & Pöschl, U., 2009. Bacteria in the global atmosphere – Part 1: Review and synthesis of literature data for different ecosystems. Atmos Chem Phys Discuss 9, 10777–10827

Buse, H.Y., Schaefer III, F.W., Rice, E.W., 2017. Enhanced survival but not amplification of *Francisella* spp. in the presence of free-living amoebae. Acta Microbiol. Immunol. Hung. 64, 17–36. doi:10.1556/030.63.2016.015

Cabezas, S., Calvete, C., Moreno, S., 2006. Vaccination success and body condition in the European wild rabbit: applications for conservation strategies. J. Wildl. Manage. 70, 1125–1131. doi:10.2193/0022-541

Cabral, M.J., 2005. In: Almeida, J., Almeida, P.R., Delliger, T., Ferrand de Almeida N., Oliveira M.E., Palmeirim J.M., Queirós A.I., Rogado L., Santos-Reis M. (eds.). Livro Vermelho dos Vertebrados de Portugal. Instituto da Conservação da Natureza. Lisboa. 659p

Callou, C., 1995. Modifications de l'aire de répartition du lapin (*Oryctolagus cuniculus*) en France et en Espagne, du Pléistocene à l'époque actuelle. État de la question. Anthropozoologica 21, 95–114

Calvete, C., 2006. Modeling the effect of population dynamics on the impact of rabbit hemorrhagic disease. Conserv. Biol. 20, 1232–41

Calvete, C., Angulo, E., Estrada, R., 2005. Conservation of European wild rabbit populations when hunting is age and sex selective. Biol. Conserv. Vol. 121; 4: 623-34

Calvete, C., Estrada, R., Angulo, E., Cabezas-Ruiz, S., 2004a. Habitat factors related to wild rabbit conservation in an agricultural landscape. Landascape Ecol. 19, 531–542

Calvete, C., Estrada, R., Lucientes, J., Osacar, J.J., Villafuerte, R., 2004b. Effects of vaccination against viral haemorrhagic disease and myxomatosis on long-term mortality rates of European wild rabbits. Vet. Rec. 155, 388–92. doi:10.1136/vr.155.13.388

Calvete, C., Estrada, R., 2004. Short-term survival and dispersal of translocated European wild rabbits. Improving the release protocol. Biol. Conserv. 120, 507–516. doi:10.1016/j.biocon.2004.03.023

Calvete, C. and Estrada, R., 2000. Epidemiología de enfermedad hemorrágica (VHD) myxomatosis en el conejo silvestre en el valle medio del Ebro. Herramientas de gestión. 1st edn. Eds Consejo de Protección de la Naturaleza. Zaragoza, ARPIrelieve. pp 77-80

Calvete, C., Villafuerte, R., Lucientes, J., Osacar, J.J., 1997. Effectiveness of traditional wild rabbit restocking in Spain. J. Zool. 241, 271–277. doi:10.1111/j.1469-7998.1997.tb01957.x

Calzada, E. de la, Martínez F. J., 1994. Requerimientos y selección de hábitat de la liebre mediterránea (*Lepus granatensis*) en un paisaje agrícola mesetario. Ecología, 8: 381-394

Camarda, A., Pugliese, N., Cavadini, P., Circella, E., Capucci, L., Caroli, A., Legretto, M., Mallia, E., Lavazza, A., 2014. Detection of the new emerging rabbit haemorrhagic disease type 2 virus (RHDV2) in Sicily from rabbit (*Oryctolagus cuniculus*) and Italian hare (*Lepus corsicanus*). Res. Vet. Sci. 97, 642–5. doi:10.1016/j.rvsc.2014.10.008

Campos R., Melo-Ferreira J., Acevedo P., 2015. Lebres fantasmas: a evolução das lebres na Península

Ibérica. CIBIO, Centro de Investigação em Biodiversidade e Recursos Genéticos. Porto. Portugal

Cancellotti, F.M., Renzi, M., 1991. Epidemiology and current situation of viral haemorrhagic disease of rabbits and the European brown hare syndrome in Italy. Rev. Sci. Tech. 10, 409–22

Capucci, L., Fallacara, F., Grazioli, S., Lavazza, A., Pacciarini, M.L., Brocchi, E., 1998. A further step in the evolution of rabbit hemorrhagic disease virus: the appearance of the first consistent antigenic variant. Virus Res. 58, 115–126. doi:10.1016/S0168-1702(98)00106-3

Capucci, L., Fusi, P., Lavazza, A., Pacciarini, M.L., Rossi, C., 1996. Detection and preliminary characterization of a new rabbit calicivirus related to rabbit hemorrhagic disease virus but nonpathogenic. J. Virol. 70, 8614–23

Capucci, L., Frigoli, G., Rønshold, L., Lavazza, A., Brocchi, E., Rossi, C., 1995. Antigenicity of the rabbit hemorrhagic disease virus studied by its reactivity with monoclonal antibodies. Virus Res. 37, 221–38.

Capucci, L., Scicluna, M.T., Lavazza, A., 1991. Diagnosis of viral haemorrhagic disease of rabbits and the European brown hare syndrome. Rev. Sci. Tech. 10, 347–70

Carro, F., J. F. Beltrán, J. M. Pérez, F. J. Márquez, O. Iborra Y R. C. Soriguer. (1999). Dinámica poblacional de la liebre ibérica (*Lepus granatensis*) en el Parque Nacional de Doñana. Resúmenes IV Jornadas SECEM, Segovia. Pp. 20

Carvalho, C.L., Duarte, E.L., Monteiro, M., Botelho, A., Albuquerque, T., Fevereiro, M., Henriques, A.M., Barros, S.S., Duarte, M.D., 2017a. Challenges in the rabbit haemorrhagic disease 2 (RHDV2) molecular diagnosis of vaccinated rabbits. Vet. Microbiol. 198, 43–50. doi:10.1016/j.vetmic.2016.12.006

Carvalho C.L., Duarte E.L., Monteiro J.M., Afonso C., Pacheco J., Carvalho P., Mendonça P., Botelho A., Albuquerque T., Themudo P., Fevereiro M., Henriques A.M., Barros S.S., Duarte M.D. 2017b. Progression of rabbit haemorrhagic disease virus 2 upon vaccination in an industrial rabbitry: a laboratorial approach. World rabbit Science . doi: https://doi.org/10.4995/wrs.2017.5708

Carvalho, C.L., Silva, S., Gouveia, P., Costa, M., Duarte, E.L., Henriques, A.M., Barros, S.S., Luís, T., Ramos, F., Fagulha, T., Fevereiro, M., Duarte, M.D., 2017c. Emergence of rabbit haemorrhagic disease virus 2 in the archipelago of Madeira, Portugal (2016–2017). Virus Genes. doi:10.1007/s11262-017-1483-6

Carvalho C.L., Zé-Zé L., Lopes de Carvalho I., Duarte E.L., 2014. Tularemia: a challenging zoonosis. Comparative Immunology and Infectious Diseases. 37(2):85-96. doi: 10.1016/j.cimid.2014.01.002

Carvalho, G.D.F., Fonseca, A., Cruz, A., Célio, P., Mântua, P., Simões, C., Silva, S., Arruda, G., 1994. Estudo Preliminar de alguns parâmetros de uma população de coelho selvagem (*Oryctolagus cuniculus*) da Ilha do Faial, Açores. Relatórios e Comunicações do Departamento de Biologia, Universidade dos Açores 22, 49–60

Carvalho, G.D.F., Ferrand, N., Fonseca, A., Branco, M., Azevedo, M., Mendes, R., Batista, P., Mântua, P., 1993. Estudo de uma população de coelhos selvagens (*Oryctolagus cuniculus*, L.) na Ilha de São Jorge, Açores. Relatórios e Comunicações do Departamento de Biologia, Universidade dos Açores 21, 8–20

Carvalho, G.D.F., Almeida, L.M.M., 1991. Contribuição para o estudo de uma populacional de coelhos selvagens (*Oryctolagus cuniculus*, L.) na Ilha de Santa Maria e o impacto do RVHD na população local

Relatórios e Comunicações do Departamento de Biologia, Universidade dos Açores 19, 61–67

Caspar, Y., Maurin, M., 2017. *Francisella tularensis* Susceptibility to Antibiotics: A Comprehensive Review of the Data Obtained In vitro and in Animal Models. Front. Cell. Infect. Microbiol. 7, 122. doi:10.3389/fcimb.2017.00122

Castañón, S., Marín, M.S., Martín-Alonso, J.M., Boga, J.A., Casais, R., Humara, J.M., Ordás, R.J., Parra, F., 1999. Immunization with potato plants expressing VP60 protein protects against rabbit hemorrhagic disease virus. J. Virol. 73, 4452–5

Catry P., Costa H., Elias G., Matias R., 2010. Aves de Portugal, Ornitologia do Território Continental. Assírio e Alvim, Lisboa

Cazenave P.A., Benammar A., Sogn J.A., Kindt T.J., 1987. Immunoglobulin genes in the feral rabbit. In: The Rabbit in Contemporary Immunological Research (ed. Dubiski S), pp. 148-163. Longman Scientific & Technical, John Wiley & Sons, New York.

Celli, J., Zahrt, T.C., 2013. Mechanisms of *Francisella tularensis* intracellular pathogenesis. Cold Spring Harb. Perspect. Med. 3, a010314. doi:10.1101/cshperspect.a010314

Centers for Disease Control and Prevention. Reported tularemia cases—United States, 2004–2013. Available at: https://www.cdc.gov/tularemia/statistics/state. html. Accessed 21 August 2015

Chasey, D., 1997. Rabbit haemorrhagic disease: the new scourge of *Oryctolagus cuniculus*. Lab. Anim. 31, 33–44. doi:10.1258/002367797780600279

Chasey, D., Lucas, M.H., Westcott, D.G., Sharp, G., Kitching, A., Hughes, S.K., 1995. Development of a diagnostic approach to the identification of rabbit haemorrhagic disease. Vet. Rec. 137, 158–60.

Chasey, D., Lucas, M., Westcott, D., Williams, M., 1992. European brown hare syndrome in the U.K.; a calicivirus related to but distinct from that of viral haemorrhagic disease in rabbits. Arch. Virol. 124, 363–370. doi:10.1007/BF01309816

Chaves, S., Neto, M., Tenreiro, R., 2009. Insect-symbiont systems: from complex relationships to biotechnological applications. Biotechnol. J. 4, 1753–65. doi:10.1002/biot.200800237

Checroun, C., Wehrly, T.D., Fischer, E.R., Hayes, S.F., Celli, J., 2006. Autophagy-mediated reentry of *Francisella tularensis* into the endocytic compartment after cytoplasmic replication. Proc. Natl. Acad. Sci. U. S. A. 103, 14578–83. doi:10.1073/pnas.0601838103

Chen, L., Liu, G., Ni, Z., Yu, B., Yun, T., Song, Y., Hua, J., Li, S., Chen, J., 2009. Minor structural protein VP2 in rabbit hemorrhagic disease virus downregulates the expression of the viral capsid protein VP60. J. Gen. Virol. 90, 2952–2955. doi:10.1099/vir.0.015081-0

Chong, A., Wehrly, T.D., Child, R., Hansen, B., Hwang, S., Virgin, H.W., Celli, J., 2012. Cytosolic clearance of replication-deficient mutants reveals *Francisella tularensis* interactions with the autophagic pathway. Autophagy 8, 1342–1356. doi:10.4161/auto.20808

Chong A, Wehrly TD, Nair V, Fischer ER, Barker JR, Klose KE, Celli J., 2008. The early phagosomal stage of *Francisella tularensis* determines optimal phagosomal escape and *Francisella* pathogenicity island protein expression. Infect Immun 76: 5488–5499

Christenson, B., 1984. An outbreak of tularemia in the northern part of central Sweden. Scand. J. Infect.

Dis. 16, 285–90

Clarke, I.N., Lambden, P.R., 1997. The molecular biology of caliciviruses. J. Gen. Virol. 78 (Pt 2), 291–301. doi:10.1099/0022-1317-78-2-291

Clay, K., Klyachko, O., Grindle, N., Civitello, D., Oleske, D., Fuqua, C., 2008. Microbial communities and interactions in the lone star tick, Amblyomma americanum. Mol. Ecol. 17, 4371–81

Clemens, D.L., Lee, B.-Y., Horwitz, M.A., 2004. Virulent and avirulent strains of *Francisella tularensis* prevent acidification and maturation of their phagosomes and escape into the cytoplasm in human macrophages. Infect. Immun. 72, 3204–17. doi:10.1128/IAI.72.6.3204-3217.2004

Cody, A.J., McCarthy, N.D., Jansen van Rensburg, M., Isinkaye, T., Bentley, S.D., Parkhill, J., Dingle, K.E., Bowler, I.C.J.W., Jolley, K.A., Maiden, M.C.J., 2013. Real-Time Genomic Epidemiological Evaluation of Human Campylobacter Isolates by Use of Whole-Genome Multilocus Sequence Typing. J. Clin. Microbiol. 51, 2526–2534. doi:10.1128/JCM.00066-13

Collery P.M., Mooney J., O'Conner M., Nowotny N., 1995. Rabbit haemorrhagic disease in Ireland Vet. Rec., 137, p. 547

Collins B.J., White J.R., Lenguas C., Boyd V., Westbury H.A., 1995. A competition ELISA for the detection of antibodies to rabbit haemorrhagic disease virus. Vet. Microbiol., 43, 85–96.

Cooke, B.D., 2002. Rabbit haemorrhagic disease: field epidemiology and the management of wild rabbit populations. Rev. Sci. Tech. 21, 347–58

Cooke B. D., A. Robinson J.,. Merchant J. C, Nardin A, Capucci L., 2000. Use of ELISAs in studies of rabbit haemorrhagic disease (RHD) in Australia Epidemiol. Infect. 124, 563±576. Printed in the United Kingdom # Cambridge University Press

Crosby, T.K. and McLennan, J., 1996. Potential vectors of rabbit calicivirus disease (RCD) in New Zealand: a review. MAF Policy. pp 29

Courchamp, F., Chapuis, J.L., Pascal, M., 2003. Mammal invaders on islands: impact, control and control impact. Biol. Rev. Camb. Philos. Soc. 78, 347–83

Curtain C.C., Wood D.H., Sobey W.R., 1973. The distribution of immunoglobulin allotypes in rabbit populations in Australia and on Macquarie Island. Animal Blood Groups and Biochemical Genetics. Vol. 4, 2: 101-109. DOI: 10.1111/j.1365-2052.1973.tb01279.x

Cypher, B.L., 1997. Effects of Radiocollars on San Joaquin Kit Foxes. J. Wildl. Manage. 61, 1412. doi:10.2307/3802145

Dale, C., Moran, N.A., 2006a. Molecular Interactions between Bacterial Symbionts and Their Hosts. Cell 126, 453–465. doi:10.1016/j.cell.2006.07.014

Dalton, K.P., Abrantes, J., Lopes, A.M., Nicieza, I., Álvarez, Á.L., Esteves, P.J., Parra, F., 2015. Complete genome sequence of two rabbit hemorrhagic disease virus variant b isolates detected on the Iberian Peninsula. Arch. Virol. 160, 877–81. doi:10.1007/s00705-014-2329-3

Dalton, K.P., Nicieza, I., Abrantes, J., Esteves, P.J., Parra, F., 2014. Spread of new variant RHDV in domestic rabbits on the Iberian Peninsula. Vet. Microbiol. 169, 67–73. doi:10.1016/j.vetmic.2013.12.015

Dalton, K.P., Nicieza, I., Balseiro, A., Muguerza, M.A., Rosell, J.M., Casais, R., Álvarez, Á.L., Parra, F., 2012.

Variant rabbit hemorrhagic disease virus in young rabbits, Spain. Emerg. Infect. Dis. 18, 2009–12. doi:10.3201/eid1812.120341

Daughenbaugh, K.F., Fraser, C.S., Hershey, J.W.B., Hardy, M.E., 2003. The genome-linked protein VPg of the Norwalk virus binds eIF3, suggesting its role in translation initiation complex recruitment. EMBO J. 22, 2852–2859. doi:10.1093/emboj/cdg251

de Carvalho, I.L., Santos, N., Soares, T., Zé-Zé, L., Núncio, M.S., 2011. *Francisella*-like endosymbiont in Dermacentor reticulatus collected in Portugal. Vector Borne Zoonotic Dis. 11, 185–8. doi:10.1089/vbz.2010.0014

Decors, A., Lesage, C., Jourdain, E., Giraud, P., Houbron, P., Vanhem, P., Madani, M., 2011. Outbreak of tularaemia in brown hares (*Lepus europaeus*) in France, January to March 2011. Eurosurveillance 16

Dee, S.A., Deen, J., Otake, S., Pijoan, C., 2004. An experimental model to evaluate the role of transport vehicles as a source of transmission of porcine reproductive and respiratory syndrome virus to susceptible pigs. Can. J. Vet. Res. 68, 128–133

De Leeuw, I., Garigliany, M., Bertels, G., Willems, T., Desmecht, D., De Clercq, K., 2015. Bluetongue virus RNA detection by real-time rt-PCR in post-vaccination samples from cattle. Transbound. Emerg. Dis. 62, 157–162. doi:http://dx.doi.org/10.1111/tbed.12100

Delibes-Mateos, M., Ferreira, C., Carro, F., Escudero, M.A., Gortázar, C., 2014. Ecosystem effects of variant rabbit hemorrhagic disease virus, Iberian Peninsula. Emerg. Infect. Dis. 20, 2166–8. doi:10.3201/eid2012.140517

Delibes-Mateos, M., Smith, A.T., Slobodchikoff, C.N., Swenson, J.E., 2011. The paradox of keystone species persecuted as pests: A call for the conservation of abundant small mammals in their native range. Biol. Conserv. 144, 1335–1346. doi:10.1016/j.biocon.2011.02.012

Delibes-Mateos, M., Farfán, M.Á., Olivero, J., Vargas, J.M., 2010. Land-use changes as a critical factor for long-term wild rabbit conservation in the Iberian Peninsula. Environ. Conserv. 37, 169–176. doi:10.1017/S0376892910000214

Delibes-Mateos, M., Ferreras, P., Villafuerte, R., 2009. European rabbit population trends and associated factors: a review of the situation in the Iberian Peninsula. Mamm. Rev. 39, 124–140. doi:10.1111/j.1365-2907.2009.00140.x

Delibes-Mateos, M., Delibes, M., Ferreras, P., Villafuerte, R., 2008. Key role of European rabbits in the conservation of the Western Mediterranean basin hotspot. Conserv. Biol. 22, 1106–17. doi:10.1111/j.1523-1739.2008.00993.x

Delibes-Mateos, M., Ferreras, P., Villafuerte, R., 2007. Rabbit populations and game management: the situation after 15 years of rabbit haemorrhagic disease in central-southern Spain. Biodivers. Conserv. 17, 559–574. doi:10.1007/s10531-007-9272-5

Dellafiore, C.M., Rouco, C., Muñóz Vallés, S., Gallego Fernández, J.B., 2014. Seasonal habitat use by the European rabbit (*Oryctolagus cuniculus*) in a coastal dune system in SW Spain. Anim. Biodivers. Conserv. 37, 233–242

Dempsey, M.P., Nietfeldt, J., Ravel, J., Hinrichs, S., Crawford, R., Benson, A.K., 2006. Paired-end sequence

mapping detects extensive genomic rearrangement and translocation during divergence of *Francisella tularensis* subsp. *tularensis* and *Francisella tularensis* subsp. *holarctica* populations. J. Bacteriol. 188, 5904–5914. doi:10.1128/JB.00437-06

Dennis, D.T., Inglesby, T. V., Henderson, D.A., Bartlett, J.G., Ascher, M.S., Eitzen, E., Fine, A.D., Friedlander, A.M., Hauer, J., Layton, M., Lillibridge, S.R., McDade, J.E., Osterholm, M.T., O'Toole, T., Parker, G., Perl, T.M., Russell, P.K., Tonat, K., 2001. Tularemia as a Biological Weapon. JAMA 285, 2763. doi:10.1001/jama.285.21.2763

Dergousoff, S.J., Chilton, N.B., 2012. Association of different genetic types of *Francisella*-like organisms with the rocky mountain wood tick (*Dermacentor andersoni*) and the American dog tick (*Dermacentor variabilis*) in localities near their northern distributional limits. Appl. Environ. Microbiol. 78, 965–71. doi:10.1128/AEM.05762-11

Desvars, A., Furberg, M., Hjertqvist, M., Vidman, L., Sjöstedt, A., Rydén, P., Johansson, A., 2015. Epidemiology and ecology of tularemia in Sweden, 1984-2012. Emerg. Infect. Dis. 21, 32–9. doi:10.3201/eid2101.140916

Didelot, X., Bowden, R., Wilson, D.J., Peto, T.E.A., Crook, D.W., 2012. Transforming clinical microbiology with bacterial genome sequencing. Nat. Rev. Genet. 13, 601–612. doi:10.1038/nrg3226

Di Modugno G., and Nasti R., 1990. La malattia emorragica virale in Puglia. Contributo sperimentale. Riv. Coniglicoltura, 27 (1), 25-32

Dobson, M., 1998. Mammal distributions in the western Mediterranean: the role of human intervention. Mamm. Rev. 28, 77–88. doi:10.1046/j.1365-2907.1998.00027.x

Douady, C.J., Delsuc, F., Boucher, Y., Doolittle, W.F., Douzery, E.J.P., 2003. Comparison of Bayesian and maximum likelihood bootstrap measures of phylogenetic reliability. Mol. Biol. Evol. 20, 248–54.

Dress A.W., Huson D.H., 2004. Constructing splits graphs. IEEE/ACM Trans Comput Biol Bioinform. Jul-Sep;1(3):109-15.

Drummond, A.J., Suchard, M.A., Xie, D., Rambaut, A., 2012. Bayesian phylogenetics with BEAUti and the BEAST 1.7. Mol. Biol. Evol. 29, 1969–1973. doi:10.1093/molbev/mss075

Drummond, A.J., Suchard, M.A., 2010. Bayesian random local clocks, or one rate to rule them all. BMC Biol. 8, 114. doi:10.1186/1741-7007-8-114

Drummond, A.J., Ho, S.Y.W., Phillips, M.J., Rambaut, A., 2006. Relaxed phylogenetics and dating with confidence. PLoS Biol. 4, 699–710. doi:10.1371/journal.pbio.0040088

Drummond, A.J., Pybus, O.G., Rambaut, A., Forsberg, R., Rodrigo, A.G., 2003. Measurably evolving populations. Trends Ecol. Evol. doi:10.1016/S0169-5347(03)00216-7

Drummond, A., Rodrigo, A.G., 2000. Reconstructing Genealogies of Serial Samples Under the Assumption of a Molecular Clock Using Serial-Sample UPGMA. Mol. Biol. Evol. 17, 1807–1815. doi:10.1093/oxfordjournals.molbev.a026281

Duarte, J., 2000. Liebre ibérica (*Lepus granatensis* Rosenhauer, 1856). Galemys 12, 3–14

Duarte, M., Carvalho, C., Bernardo, S., Barros, S.V., Benevides, S., Flor, L., Monteiro, M., Marques, I., Henriques, M., Barros, S.C., Fagulha, T., Ramos, F., Luís, T., Fevereiro, M., 2015a. Rabbit haemorrhagic

disease virus 2 (RHDV2) outbreak in Azores: Disclosure of common genetic markers and phylogenetic segregation within the European strains. Infect. Genet. Evol. 35, 163–71. doi:10.1016/j.meegid.2015.08.005

Duarte, M., Henriques, M., Barros, S.C., Fagulha, T., Ramos, F., Luís, T., Fevereiro, M., Benevides, S., Flor, L., Barros, S.V., Bernardo, S., 2015b. Detection of RHDV variant 2 in the Azores. Vet. Rec. 176, 130. doi:10.1136/vr.h497

Duarte, M.D., Henriques, A.M., Barros, S., Luís, T., Fagulha, T., Ramos, F., Fevereiro, M., 2014. New insight into the epidemiology of rabbit hemorrhagic disease viruses in Portugal: retrospective study reveals the circulation of genogroup 5 (G5) in Azores and discloses the circulation of G1 and G6 strains in mainland until 2008. Infect. Genet. Evol. 27, 149–55. doi:10.1016/j.meegid.2014.07.017

Duron, O., Noël, V., McCoy, K.D., Bonazzi, M., Sidi-Boumedine, K., Morel, O., Vavre, F., Zenner, L., Jourdain, E., Durand, P., Arnathau, C., Renaud, F., Trape, J.-F., Biguezoton, A.S., Cremaschi, J., Dietrich, M., Léger, E., Appelgren, A., Dupraz, M., Gómez-Díaz, E., Diatta, G., Dayo, G.-K., Adakal, H., Zoungrana, S., Vial, L., Chevillon, C., 2015. The Recent Evolution of a Maternally-Inherited Endosymbiont of Ticks Led to the Emergence of the Q Fever Pathogen, Coxiella burnetii. PLoS Pathog. 11, e1004892. doi:10.1371/journal.ppat.1004892

Duron, O., 2014. *Arsenophonus* insect symbionts are commonly infected with APSE, a bacteriophage involved in protective symbiosis. FEMS Microbiol. Ecol. 90, 184–94. doi:10.1111/1574-6941.12381

Dwibedi, C., Birdsell, D., Lärkeryd, A., Myrtennäs, K., Öhrman, C., Nilsson, E., Karlsson, E., Hochhalter, C., Rivera, A., Maltinsky, S., Bayer, B., Keim, P., Scholz, H.C., Tomaso, H., Wittwer, M., Beuret, C., Schuerch, N., Pilo, P., Hernández Pérez, M., Rodriguez-Lazaro, D., Escudero, R., Anda, P., Forsman, M., Wagner, D.M., Larsson, P., Johansson, A., 2016. Long-range dispersal moved *Francisella tularensis* into Western Europe from the East. Microb. Genomics 2, e000100. doi:10.1099/mgen.0.000100

Eden, J.-S., Kovaliski, J., Duckworth, J.A., Swain, G., Mahar, J.E., Strive, T., Holmes, E.C., 2015. Comparative Phylodynamics of Rabbit Hemorrhagic Disease Virus in Australia and New Zealand. J. Virol. 89, 9548–9558. doi:10.1128/JVI.01100-15

Ellis, J., Oyston, P.C.F., Green, M., Titball, R.W., 2002. Tularemia. Clin. Microbiol. Rev. doi:10.1128/CMR.15.4.631-646.2002

Elsworth, P., Cooke, B.D., Kovaliski, J., Sinclair, R., Holmes, E.C., Strive, T., 2014. Increased virulence of rabbit haemorrhagic disease virus associated with genetic resistance in wild Australian rabbits (*Oryctolagus cuniculus*). Virology 464-465, 415–423. doi:10.1016/j.virol.2014.06.037

Erber M, Gerbermann H, Meiler H, Schindlmayr R (1991) Rabbit Haemorrhagic Disease (RHD) bei Hauskaninchen in Bayern: epidemiologische, pathomorphologische und virologische Untersuchungen. Tierirztl Umschau46, 151-155

Eremkin, A. V., Elagin, G.D., Petchenkin, D. V., Fomenkov, O.O., Bogatcheva, N. V., Kitmanov, A. A., Kuklina, G. V., Tikhvinskaya, O. V., 2016. The development of immune enzyme and immune chromatographic monoclonal test-system for detecting tularemia agent. Klin. Lab. Diagn. 61, 184–7. doi:10.18821/0869-2084-2016-61-3-184-187

Escudero, R., Toledo, A., Gil, H., Kovácsová, K., Rodríguez-Vargas, M., Jado, I., García-Amil, C., Lobo, B., Bhide, M., Anda, P., 2008. Molecular method for discrimination between *Francisella tularensis* and *Francisella*-like endosymbionts. J. Clin. Microbiol. 46, 3139–43. doi:10.1128/JCM.00275-08

Esteves, P.J., Abrantes, J., Bertagnoli, S., Cavadini, P., Gavier-Widén, D., Guitton, J.S., Lavazza, A., Lemaitre, E., Letty, J., Lopes, A.M., Neimanis, A.S., Ruvoën-Clouet, N., Le Pendu, J., Marchandeau, S., Le Gall-Reculé, G., 2015. Emergence of Pathogenicity in Lagoviruses: Evolution from Pre-existing Nonpathogenic Strains or through a Species Jump? PLoS Pathog. 11, e1005087. doi:10.1371/journal.ppat.1005087

Esteves, P.J., Lopes, A.M., Magalhães, M.J., Pinheiro, A., Gonçalves, D., Abrantes, J., 2014. Rabbit hemorrhagic disease virus detected in Pico, Azores, Portugal, revealed a unique endemic strain with more than 17 years of independent evolution. Viruses 6, 2698–707. doi:10.3390/v6072698

Esteves, P.J., Abrantes, J., Carneiro, M., Müller, A., Thompson, G., Van Der Loo, W., 2008. Detection of positive selection in the major capsid protein VP60 of the rabbit haemorrhagic disease virus (RHDV). Virus Res. 137, 253–256. doi:10.1016/j.virusres.2008.07.025

Esteves, P.J., Lanning, D., Ferrand, N., Knight, K.L., Zhai, S.-K., van der Loo, W., 2004. Allelic variation at the VHa locus in natural populations of rabbit (*Oryctolagus cuniculus*, L.). J. Immunol. 172, 1044–53.

Farfán, M. Á., Guerrero, J. C., Vargas, J. M., Barbosa, A. M., Real, R., 2004. Predictive models of hunting yield distribution for the wild rabbit (*Oryctolagus cuniculus*) and the Iberian hare (*Lepus granatensis*) in Andalusia (South of Spain). Research Center in Biodiversity and Genetic Resources, Vairão.

Farlow, J., Wagner, D.M., Dukerich, M., Stanley, M., Chu, M., Kubota, K., Petersen, J., Keim, P., 2005. *Francisella tularensis* in the United States. Emerg. Infect. Dis. 11, 1835–41. doi:10.3201/eid1112.050728

Farnós, O., Fernández, E., Chiong, M., Parra, F., Joglar, M., Méndez, L., Rodríguez, E., Moya, G., Rodríguez, D., Lleonart, R., González, E.M., Alonso, A., Alfonso, P., Suárez, M., Rodríguez, M.P., Toledo, J.R., 2009. Biochemical and structural characterization of RHDV capsid protein variants produced in *Pichia pastoris*: Advantages for immunization strategies and vaccine implementation. Antiviral Res. 81, 25–36. doi:10.1016/j.antiviral.2008.08.007

Farnós, O., Rodríguez, D., Valdés, O., Chiong, M., Parra, F., Toledo, J.R., Fernández, E., Lleonart, R., Suárez, M., 2007. Molecular and antigenic characterization of rabbit hemorrhagic disease virus isolated in Cuba indicates a distinct antigenic subtype. Arch. Virol. 152, 1215–21. doi:10.1007/s00705-006-0926-5

Farnós, O., Boué, O., Parra, F., Martín-Alonso, J.M., Valdés, O., Joglar, M., Navea, L., Naranjo, P., Lleonart, R., 2005. High-level expression and immunogenic properties of the recombinant rabbit hemorrhagic disease virus VP60 capsid protein obtained in *Pichia pastoris*. J. Biotechnol. 117, 215–224. doi:10.1016/j.jbiotec.2005.01.013

Feldman, K.A., Enscore, R.E., Lathrop, S.L., Matyas, B.T., McGuill, M., Schriefer, M.E., Stiles-Enos, D., Dennis, D.T., Petersen, L.R., Hayes, E.B., 2001. An Outbreak of Primary Pneumonic Tularemia on Martha's Vineyard. N. Engl. J. Med. 345, 1601–1606. doi:10.1056/NEJMoa011374

Felsenstein, J., 1985. Confidence limits on phylogenies: An approach using the bootstrap. Evolution 39, 783–791. doi:10.1111/j.1558-5646.1985.tb00420.x

Felsenstein, J., 1981. Evolutionary trees from DNA sequences: A maximum likelihood approach. J. Mol. Evol. 17, 368–376. doi:10.1007/BF01734359

Felsenstein, J., 1973. Maximum likelihood and minimum-steps methods for estimating evolutionary trees from data on discrete characters. Syst. Biol. 22, 240–249. doi:10.1093/sysbio/22.3.240

Fenner, F., Marshall, I.D., 1957. A comparison of the virulence for European rabbits (*Oryctolagus cuniculus*) of strains of myxoma virus recovered in the field in Australia, Europe and America. J. Hyg. (Lond). 55, 149–91. doi:10.1017/S0022172400037098

Fenner, F.; Ratcliffe, FN. 1965. Myxomatosis. Cambridge University Press

Fernández, E., Toledo, J.R., Chiong, M., Parra, F., Rodríguez, E., Montero, C., Méndez, L., Capucci, L., Farnós, O., 2011. Single dose adenovirus vectored vaccine induces a potent and long-lasting immune response against rabbit hemorrhagic disease virus after parenteral or mucosal administration. Vet Immunol Immunopathol. 2011 Aug 15;142(3-4):179-88. doi: 10.1016/j.vetimm.2011.05.007. Epub 2011 May 7

Fernandez, A., Soriguer, R., Castien, E., Carro, F., 2008. Reproduction parameters of the Iberian hare *Lepus granatensis* at the edge of its range. Wildlife Biol. 14, 434–443. doi:10.2981/0909-6396-14.4.434

Fernandez, A., Soriguer, R., Carro, F. and Castien, E. 2004. Population trends of two hare species (*Lepus granatensis* and *Lepus europaeus*) present in Navarra province (northern Iberian Peninsula) over the last two decades. Research Center in Biodiversity and Genetic Resources, Vairao.

Fernández-Fernández, M.R., Mouriño, M., Rivera, J., Rodríguez, F., Plana-Durán, J., García, J.A., 2001. Protection of Rabbits against Rabbit Hemorrhagic Disease Virus by Immunization with the VP60 Protein Expressed in Plants with a Potyvirus-Based Vector. Virology 280, 283–291. doi:10.1006/viro.2000.0762

Ferrand, N., 2008. Inferring the evolutionary history of the European rabbit (*Oryctolagus cuniculus*) from molecular markers. In: Alves P.C., Ferrand N., Hackländer K. (eds) Lagomorph Biology. Springer, Berlin, Heidelberg. https://doi.org/10.1007/978-3-540-72446-9_4

Ferrand, N., Branco, M., 2007. The evolutionary history of the European rabbit (*Oryctolagus cuniculus*): major patterns of population differentiation and geographic expansion inferred from protein polymorphism, in: Phylogeography of Southern European Refugia. Springer Netherlands, Dordrecht, pp. 207–235. doi:10.1007/1-4020-4904-8_7

Ferreira, A., Ferreira, A.J., 2014. Post-weaning growth of endemic Iberian wild rabbit subspecies, *Orycto lagus cuniculus algirus*, kept in a semi-extensive enclosure : implications for management and conservation. World Rabbit Sci. 22, 129. doi:10.4995/wrs.2014.1673

Ferreira, C., 2012. European rabbit research in the Iberian Peninsula: state of the art and future perspectives. Eur. J. Wildl. Res. 58:885. https://doi.org/10.1007/s10344-012-0664-9

Ferreira, C., Alves, P., 2009. Influence of habitat management on the abundance and diet of wild rabbit

(*Oryctolagus cuniculus algirus*) populations in Mediterranean ecosystems. Eur. J. Wildl. Res. 55: 487. https://doi.org/10.1007/s10344-009-0257-4

Ferreira, C.M.A. de C., Delibes-Mateos, M., 2010. Wild rabbit management in the iberian peninsula: state of the art and future perspectives for iberian lynx conservation. Wildl. Biol. Pract. 6. doi:10.2461/wbp.lynx.4

Ferreira, P.G., Costa-E-Silva, A., Oliveira, M.J.R., Monteiro, E., Águas, A.P., 2005. Leukocyte–hepatocyte interaction in calicivirus infection: differences between rabbits that are resistant or susceptible to rabbit haemorrhagic disease (RHD). Vet. Immunol. Immunopathol. 103, 217–221. doi:10.1016/j.vetimm.2004.09.028

Ferreira, P.G., Costa-e-Silva, A., Monteiro, E., Oliveira, M.J.R., Águas, A.P., 2004. Transient decrease in blood heterophils and sustained liver damage caused by calicivirus infection of young rabbits that are naturally resistant to rabbit haemorrhagic disease. Res. Vet. Sci. 76, 83–94

Ferretti, M., Paci, G., Porrini, S., Galardi, L., Bagliacca, M., 2010. Habitat use and home range traits of resident and relocated hares (*Lepus europaeus*, Pallas). Ital. J. Anim. Sci. 9, e54. doi:10.4081/ijas.2010.e54

Fischer, L., Le Gros, F.X., Mason, P.W., Paoletti, E., 1997. A recombinant canarypox virus protects rabbits against a lethal rabbit hemorrhagic disease virus (RHDV) challenge. Vaccine 15, 90–96. doi:10.1016/S0264-410X(96)00102-8

Fitzner A., Niedbalski W., Kęsy A., Paprocka G., 2011. Detection of RHD virus by a real-time reverse transcription PCR. Bull Vet Inst Pulawy 55, 581-586

Flux, J.E.C., Fullagar, P.J., 1992. World distribution of the rabbit *Oryctolagus cuniculus* on islands. Mamm. Rev. 22, 151–205. doi:10.1111/j.1365-2907.1992.tb00129.x

Flux, J., Angermann, R., 1990: Introduction and overview of the order *Lagomorpha*. In: Chapman, J.A. & Flux,J.(Eds.);Rabbits,HaresandPikas.StatusSurvey and Conservation Action Plan. IUCN/WWF, pp. 1-7

Forrester, N.L., Moss, S.R., Turner, S.L., Schirrmeier, H., Gould, E.A., 2008. Recombination in rabbit haemorrhagic disease virus: Possible impact on evolution and epidemiology. Virology 376, 390–396. doi:10.1016/j.virol.2008.03.023

Forrester, N.L., Trout, R.C., Gould, E.A., 2007. Benign circulation of rabbit haemorrhagic disease virus on Lambay Island, Eire. Virology 358, 18–22. doi:10.1016/j.virol.2006.09.011

Forrester, N.L., Trout, R.C., Turner, S.L., Kelly, D., Boag, B., Moss, S., Gould, E.A., 2006a. Unravelling the paradox of rabbit haemorrhagic disease virus emergence, using phylogenetic analysis; possible implications for rabbit conservation strategies. Biol. Conserv. 131, 296–306. doi:10.1016/j.biocon.2006.05.005

Forrester, N.L., Abubakr, M.I., Abu Elzein, E.M.E., Al-Afaleq, A.I., Housawi, F.M.T., Moss, S.R., Turner, S.L., Gould, E.A., 2006b. Phylogenetic analysis of Rabbit haemorrhagic disease virus strains from the Arabian Peninsula: Did RHDV emerge simultaneously in Europe and Asia? Virology 344, 277–282. doi:10.1016/j.virol.2005.10.006

Fouchet, D., Le Pendu, J., Guitton, J.S., Guiserix, M., Marchandeau, S., Pontier, D., 2009. Evolution of

microparasites in spatially and genetically structured host populations: the example of RHDV infecting rabbits. J. Theor. Biol. 257, 212–27. doi:10.1016/j.jtbi.2008.11.021

Frescura T., Gialletti L., Rutili D., Fioroni A., Marini C., de Mia G.M., 1989. Malattia emorragica virale del coniglio. Obiet. Docum. vet., 2 , 37-40

Frölich, K., Klima, F., Dedek, J., 1998. Antibodies against rabbit hemorrhagic disease virus in free-ranging red foxes from Germany. J. Wildl. Dis. 34, 436–42. doi:10.7589/0090-3558-34.3.436

Fujita, O., Uda, A., Hotta, A., Okutani, A., Inoue, S., Tanabayashi, K., Yamada, A., 2008. Genetic diversity of *Francisella tularensis* subspecies *holarctica* strains isolated in Japan. Microbiol. Immunol. 52, 270–6. doi:10.1111/j.1348-0421.2008.00036.x

Fuller, J.R., Craven, R.R., Hall, J.D., Kijek, T.M., Taft-Benz, S., Kawula, T.H., 2008. RipA, a cytoplasmic membrane protein conserved among *Francisella species*, is required for intracellular survival. Infect. Immun. 76, 4934–4943. doi:10.1128/IAI.00475-08

Gall A., Hoffmann B., Teifke J.P., Lange B, Schirrmeier H. 2007. Persistence of viral RNA in rabbits which overcome an experimental RHDV infection detected by a highly sensitive multiplex real-time RT-PCR. Vet Microbiol. Feb 25;120(1-2):17-32. Epub 2006 Oct 12

Gall, A., Schirrmeier, H., 2006. Persistence of rabbit haemorrhagic disease virus genome in vaccinated rabbits after experimental infection. J. Vet. Med. B. Infect. Dis. Vet. Public Health 53, 358–62. doi:10.1111/j.1439-0450.2006.00986.x

Gálvez, L., López-Pintor, A., De Miguel, J.M., Alonso, G., Rueda, M., Rebollo, S., Gómez-Sal, A., 2008. Ecosystem Engineering Effects of European Rabbits in a Mediterranean Habitat, in: Lagomorph Biology. Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 125–139. doi:10.1007/978-3-540-72446-9_9

Gao, J., Meng, C., Chen, Z., Li, C., Liu, G., 2013. Codon optimization of the rabbit hemorrhagic disease virus (RHDV) capsid gene leads to increased gene expression in *Spodoptera frugiperda* 9 (*Sf*9) cells. J. Vet. Sci. 14, 441–7. doi:10.4142/jvs.2013.14.4.441

Gaur, R., Alam, S.I., Kamboj, D.V., 2017. Immunoproteomic Analysis of Antibody Response of Rabbit Host Against Heat-Killed *Francisella tularensis* Live Vaccine Strain. Curr. Microbiol. 74, 499–507. doi:10.1007/s00284-017-1217-y

Gavier-Widén D., Morner T., 1993. Descriptive epizootiological study of European brown hare syndrome in Sweden. J Wildl Dis, 29:15-20

Gavier-Widén, D., Mörner, T., 1991. Epidemiology and diagnosis of the European brown hare syndrome in Scandinavian countries: a review. Rev. Sci. Tech. 10, 453–8

Gehrmann, B., Kretzschmar, C., 1991. An experimental contribution to the epizootiology of viral hemorrhagic septicemia of rabbits (rabbit hemorrhagic disease, RHD)-transmission by flies. Berl. Munch. Tierarztl. Wochenschr. 104, 192–194

Geier H., Celli J., 2011. Phagocytic Receptors Dictate Phagosomal Escape and Intracellular Proliferation of *Francisella tularensis*. Infect Immun. Jun; 79(6): 2204–2214. doi: 10.1128/IAI.01382-10

Gelmetti D., Grieco V., Rossi C., Capucci L., Lavazza A., 1998. Detection of rabbit haemorrhagic disease

virus (RHDV) by *in situ* hybridisation with a digoxigenin labelled RNA probe. J Virol Methods. Jun;72(2):219-26

Gerhart, J.G., Moses, A.S., Raghavan, R., 2016. A *Francisella*-like endosymbiont in the Gulf Coast tick evolved from a mammalian pathogen. Sci. Rep. 6, 33670. doi:10.1038/srep33670

Gibb J.A. 1990. The European Rabbit. In: Chapman J.A., Flux J.E. (eds) Rabbits, hares and pikas: status survey and conservation action plan. IUCN, Switzerland, 116-120

Gibb J. A., White A. J., C. P. Ward., 1985.Population ecology of rabbits in the wairarapa, New Zealand. New Zealand Journal of Ecology. Vol. 8, pp. 55-82

Gil F., Titarenko E., Terrada E., Arcalís E., Escribano J.M., 2006. Successful oral prime-immunization with VP60 from rabbit haemorrhagic disease virus produced in transgenic plants using different fusion strategies. Plant Biotechnol J. 4(1):135-43

Glass, P.J., White, L.J., Ball, J.M., Leparc-Goffart, I., Hardy, M.E., Estes, M.K., 2000. Norwalk virus open reading frame 3 encodes a minor structural protein. J. Virol. 74, 6581–91

Goethert, H.K., Telford, S.R., 2009. Nonrandom distribution of vector ticks (*Dermacentor variabilis*) infected by Francisella tularensis. PLoS Pathog. 5, e1000319. doi:10.1371/journal.ppat.1000319

Goethert, H.K., Telford, S.R., 2005. A new Francisella (Beggiatiales: Francisellaceae) inquiline within Dermacentor variabilis say (Acari: Ixodidae). J. Med. Entomol. 42, 502–5

Golovliov, I., Lindgren, H., Eneslätt, K., Conlan, W., Mosnier, A., Henry, T., Sjöstedt, A., 2016. An i*n vitro* co-culture mouse model demonstrates efficient vaccine-mediated control of *Francisella tularensis* SCHU S4 and identifies nitric oxide as a predictor of efficacy. Front. Cell. Infect. Microbiol. 6, 152. doi:10.3389/fcimb.2016.00152

Goodfellow, I., Chaudhry, Y., Gioldasi, I., Gerondopoulos, A., Natoni, A., Labrie, L., Laliberté, J.-F., Roberts, L., 2005. Calicivirus translation initiation requires an interaction between VPg and eIF 4 E. EMBO Rep. 6, 968–972. doi:10.1038/sj.embor.7400510

Gortazar, C., Millán, J., Acevedo, P., Escudero, M.A., Marco, J., de Luco, D.F., 2007. A large-scale survey of brown hare *Lepus europaeus* and Iberian hare *L. granatensis* populations at the limit of their ranges. Wildlife Biol. 13, 244–250. doi:10.2981/0909-6396(2007)13[244:ALSOBH]2.0.CO;2

Gorvel, J.P., Rigal, A., Sarles, J., Maroux, S., 1985. Aminopeptidase N- and human blood group A-antigenicity along the digestive tract and associated glands in the rabbit. Cell Tissue Res. 239, 241–248. doi:10.1007/BF00214925

Granzow, H., Weiland, F., Strebelow, H.-G., Liu, C.., Schirrmeier, H., 1996. Rabbit hemorrhagic disease virus (RHDV): ultrastructure and biochemical studies of typical and core-like particles present in liver homogenates. Virus Res. 41, 163–172. doi:10.1016/0168-1702(96)01285-3

Grazioli S, Agnoletti F, Scicluna MT, Masoero N, Guercio A, Fallacara F, Lavazza A, Brocchi E, Capucci L (2000) Rabbit haemorrhagic disease virus (RHDV) subtype "A" (RHDVa) is replacing the original strain in some Italian regions. In: Brocchi E, Lavazza A (eds), Fifth International

Gregg, D.A., House, C., Meyer, R., Berninger, M., 1991. Viral haemorrhagic disease of rabbits in Mexico: epidemiology and viral characterization. Rev. Sci. Tech. 10, 435–51

Gregory, S.H., Mott, S., Phung, J., Lee, J., Moise, L., McMurry, J.A., Martin, W., De Groot, A.S., 2009. Epitope-based vaccination against pneumonic tularemia. Vaccine 27, 5299–5306. doi:10.1016/j.vaccine.2009.06.101

Grossman, T.H., Anderson, M.S., Christ, D., Gooldy, M., Henning, L.N., Heine, H.S., Kindt, M.V., Lin, W., Siefkas-Patterson, K., Radcliff, A.K., Tam, V.H., Sutcliffe, J.A., 2017. The fluorocycline TP-271 is efficacious in models of aerosolized *Francisella tularensis* SCHU S4 infection in BALB/c mice and cynomolgus macaques, Antimicrobial Agents and Chemotherapy. doi:10.1128/AAC.00448-17

Gubernot, D.M., Boyer, B.L., Moses, M.S., 2008. Animals as early detectors of bioevents: veterinary tools and a framework for animal-human integrated zoonotic disease surveillance. Public Health Rep. 123, 300–15. doi:10.1177/003335490812300310

Guerrero-Casado J., Ruiz-Aizpurua L., Carpio A.J., Tortosa F.S., 2013. Factors affecting wild rabbit production in extensive breeding enclosures: how can we optimise efforts? World Rabbit Sci., 21: 193-199. doi:10.4995/wrs.2013.1259

Guillon, P., Ruvoën-clouet, N., Le Moullac-Vaidye, B., Marchandeau, S., Le Pendu, J., 2009. Association between expression of the H histo-blood group antigen, α1,2fucosyltransferases polymorphism of wild rabbits, and sensitivity to rabbit hemorrhagic disease virus. Glycobiology 19, 21–28. doi:10.1093/glycob/cwn098

Guittré C., Baginski I., Le Gall G., Prave M., Trepo C., Cova L., 1995. Detection of rabbit haemorrhagic disease virus isolates and sequence comparison of the N-terminus of the capsid protein gene by the polymerase chain reaction. Res Vet Sci. 58:128–132. doi: 10.1016/0034-5288(95)90065-9

Guittré C, Ruvoen-Clouet N, Barraud L, Cherel Y, Baginski I, Prave M, Ganiere JP, Trépo C, Cova. Early stages of rabbit haemorrhagic disease virus infection monitored by polymerase chain reaction. Zentralbl Veterinarmed B. 1996 Apr;43(2):109-18

Guix, S., Asanaka, M., Katayama, K., Crawford, S.E., Neill, F.H., Atmar, R.L., Estes, M.K., 2007. Norwalk Virus RNA Is Infectious in Mammalian Cells. J. Virol. 81, 12238–12248. doi:10.1128/JVI.01489-07

Gutiérrez, M.P., Bratos, M.A., Garrote, J.I., Dueñas, A., Almaraz, A., Alamo, R., Rodríguez Marcos, H., Rodríguez Recio, M.J., Muñoz, M.F., Orduña, A., Rodríguez-Torres, A., 2003. Serologic evidence of human infection by *Francisella tularensis* in the population of Castilla y León (Spain) prior to 1997. FEMS Immunol. Med. Microbiol. 35, 165–169. doi:10.1016/S0928-8244(03)00002-6

Gyuranecz, M., Birdsell, D.N., Splettstoesser, W., Seibold, E., Beckstrom-Sternberg, S.M., Makrai, L., Fodor, L., Fabbi, M., Vicari, N., Johansson, A., Busch, J.D., Vogler, A.J., Keim, P., Wagner, D.M., 2012. Phylogeography of *Francisella tularensis* subsp. *holarctica* , Europe. Emerg. Infect. Dis. 18, 290–293. doi:10.3201/eid1802.111305

Gyuranecz, M., Szeredi, L., Makrai, L., Fodor, L., Mészáros, Á.R., Szépe, B., Füleki, M., Erdélyi, K., 2010. Tularemia of European brown hare (*Lepus europaeus*). Vet. Pathol. Online 47, 958–963. doi:10.1177/0300985810369902

Hall, R.N., Mahar, J.E., Haboury, S., Stevens, V., Holmes, E.C., Strive, T., 2015. Emerging Rabbit Hemorrhagic Disease Virus 2 (RHDVb), Australia. Emerg. Infect. Dis. 21, 2276–8.

doi:10.3201/eid2112.151210

Hanchun, Y., Xu, W.Y. & Du, N. (1991), Hemagglutination characteristics of rabbit hemorrhagic disease virus. Proceedings of International Symposium on Rabbit Haemorrhagic Disease. August 6-10. Beijing, People's Republica of China. Koscielak, J., Zderbska, E., Wilczy

Harrington P.R., Lindensmith L., Yount B., Moe C.L., Baric R.S., 2002. Binding of Norwalk virus-like particles to ABH histo-blood group antigens is blocked by antisera from infected human volunteers or experimentally vaccinated mice, J Virol. vol. 76 (pg. 12325-12343)

Harrison, S.C., 2001. Principles of virus structure. *In* Knipe, D.M., Howley, P.M., Griffin, D.E., Martin, M.A., Lamb, R.A., Roizman, B., Strauss, S.E. (Eds.), 4th ed. Fields Virology, vol. 1. Lippincott Williams & Wilkins, Philadelphia, PA, pp. 53-85

Hastings, W.K., 1970. Monte Carlo Sampling Methods Using Markov Chains and Their Applications. Biometrika 57, 97. doi:10.2307/2334940

Hazelton, P.R., Gelderblom, H.R., 2003. Electron microscopy for rapid diagnosis of infectious agents in emergent situations. Emerg. Infect. Dis. 9, 294–303. doi:10.3201/eid0903.020327

Henning, J., Meers, J., Davies, P.R., Morris, R.S., 2005. Survival of rabbit haemorrhagic disease virus (RHDV) in the environment. Epidemiol. Infect. 133, 719–30.

Henry, T., Brotcke, A., Weiss, D.S., Thompson, L.J., Monack, D.M., 2007. Type I interferon signaling is required for activation of the inflammasome during Francisella infection. J. Exp. Med. 204, 987–994. doi:10.1084/jem.20062665

Hickey, A.J., Hazlett, K.R.O., Kirimanjeswara, G.S., Metzger, D.W., 2011. Identification of *Francisella tularensis* outer membrane protein A (FopA) as a protective antigen for tularemia. Vaccine 29, 6941–6947. doi:10.1016/j.vaccine.2011.07.075

Hicks, A.L., Duffy, S., 2012. One misdated sequence of rabbit hemorrhagic disease virus prevents accurate estimation of its nucleotide substitution rate. BMC Evol. Biol. 12, 74. doi:10.1186/1471-2148-12-74

Holmes, E.C., 2013. What can we predict about viral evolution and emergence? Curr. Opin. Virol. 3, 180–184. doi:10.1016/j.coviro.2012.12.003

Hu, Z., Tian, X., Zhai, Y., Xu, W., Zheng, D., Sun, F., 2010. Cryo-electron microscopy reconstructions of two types of wild rabbit hemorrhagic disease viruses characterized the structural features of *Lagovirus*. Protein Cell 1, 48–58. doi:10.1007/s13238-010-0007-0

Huang, H.B., 1991. Vaccination against and immune response to viral haemorrhagic disease of rabbits: a review of research in the People's Republic of China. Rev. Sci. Tech. 10, 481–98

Huang B.K., 1986. Observations on emergency inoculation of infected rabbits (*in Chinese*). Chin. J. vet. Sci. Technol., 16 (3), 33-34

Hubálek, Z., Rudolf, I., 2017. *Francisella tularensis* prevalence and load in *Dermacentor reticulatus* ticks in an endemic area in Central Europe. Med. Vet. Entomol. 31, 234–239. doi:10.1111/mve.12229

Huber K.T., Van Iersel L., Moulton V., Wu T., 2015. How much information is needed to infer reticulate evolutionary histories? Syst Biol. Jan;64(1):102-11. doi: 10.1093/sysbio/syu076. Epub 2014Sep 18

Huelsenbeck, J. P., B. Larget, R. E. Miller, Ronquist F., 2002. Potential applications and pitfalls of Bayesian inference of phylogeny. Syst. Biol. 51:673–688

Huelsenbeck, J.P., Ronquist, F., Nielsen, R., Bollback, J.P., 2001. Bayesian inference of phylogeny and its impact on evolutionary biology. Science (80-.). 294, 2310–2314. doi:10.1126/science.1065889

Huelsenbeck, J.P., Larget, B., Swofford, D., 2000. A compound Poisson process for relaxing the molecular clock. Genetics 154, 1879–1892. doi:10.1016/j.tig.2005.04.001

Huelsenbeck, J.P., 1995. The robustness of two phylogenetic methods: four-taxon simulations reveal a slight superiority of maximum likelihood over neighbor joining. Mol. Biol. Evol. 12, 843–9

Huntley, J.F., Conley, P.G., Rasko, D.A., Hagman, K.E., Apicella, M.A., Norgard, M. V., 2008. Native outer membrane proteins protect mice against pulmonary challenge with virulent type A *Francisella tularensis*. Infect. Immun. 76, 3664–3671. doi:10.1128/IAI.00374-08

Huson, D.H., Bryant, D., 2006. Application of phylogenetic networks in evolutionary studies. Mol. Biol. Evol. doi:10.1093/molbev/msj030

Huson, D.H., Rupp, R., Scornavacca, C., 2010. Phylogenetic Networks: Concepts, Algorithms and Applications (Google eBook). Cambridge University Press

Huson DH, Scornavacca C. 2010. A survey of combinatorial methods for phylogenetic networks. Genome Biol Evol 3: 23–35

Hutt, J.A., Lovchik, J.A., Dekonenko, A., Hahn, A.C., Wu, T.H., 2017. The natural history of pneumonic tularemia in female fischer 344 rats after inhalational exposure to aerosolized *francisella tularensis* subspecies *tularensis* strain SCHU S4. Am. J. Pathol. 187, 252–267. doi:10.1016/j.ajpath.2016.09.021

Ivanov, I.N., Mitkova, N., Reye, A.L., Hübschen, J.M., Vatcheva-Dobrevska, R.S., Dobreva, E.G., Kantardjiev, T. V, Muller, C.P., 2011. Detection of new *Francisella*-like tick endosymbionts in *Hyalomma* spp. and *Rhipicephalus* spp. (Acari: Ixodidae) from Bulgaria. Appl. Environ. Microbiol. 77, 5562–5. doi:10.1128/AEM.02934-10

Jahnke, M., Holmes, E.C., Kerr, P.J., Wright, J.D., Strive, T., 2010. Evolution and Phylogeography of the Nonpathogenic Calicivirus RCV-A1 in Wild Rabbits in Australia. J. Virol. 84, 12397–12404. doi:10.1128/JVI.00777-10

Johansson, A., Farlow, J., Larsson, P., Dukerich, M., Chambers, E., Byström, M., Fox, J., Chu, M., Forsman, M., Sjöstedt, A., Keim, P., 2004. Worldwide genetic relationships among *Francisella tularensis* isolates determined by multiple-locus variable-number tandem repeat analysis. J. Bacteriol. 186, 5808–5818. doi:10.1128/JB.186.17.5808-5818.2004

Jones, J.W., Broz, P., Monack, D.M., 2011. Innate immune recognition of *Francisella tularensis*: activation of Type-I interferons and the inflammasome. Front. Microbiol. 2, 16. doi:10.3389/fmicb.2011.00016

Jung, J.Y., Lee, B.J., Tai, J.., Park, J.., Lee, Y.S., 2000. Apoptosis in rabbit haemorrhagic disease. J. Comp. Pathol. 123, 135–140. doi:10.1053/jcpa.2000.0403

Karhukorpi, E.K., Karhukorpi, J., 2001. Rapid laboratory diagnosis of ulceroglandular tularemia with polymerase chain reaction. Scand. J. Infect. Dis. 33, 383–5

Karlsson, E., Golovliov, I., Lärkeryd, A., Granberg, M., Larsson, E., Öhrman, C., Niemcewicz, M., Birdsell, D.,

Wagner, D.M., Forsman, M., Johansson, A., 2016. Clonality of erythromycin resistance in *Francisella tularensis*. J. Antimicrob. Chemother. 71, 2815–23. doi:10.1093/jac/dkw235

Kaushal, A., Gupta, K., Shah, R., van Hoek, M.L., 2016. Antimicrobial activity of mosquito cecropin peptides against *Francisella*. Dev. Comp. Immunol. 63, 171–180. doi:10.1016/j.dci.2016.05.018

Keim P., Johansson A., Wagner D.M., 2007. Molecular epidemiology, evolution, and ecology of *Francisella*. Ann N Y Acad Sci.;1105:30–66. http://dx.doi.org/10.1196/annals.1409.011

Romanova, L. V., Mishan'kin, B. N., Pichurina, N. L., Vodop'ianov, S. O., Saiamov, S. R., 2000. Noncultivatable forms of *Francisella tularensis*. Zh Mikrobiol Epidemiol Immunobiol, 11–15

Kenney, A., Cusick, A., Payne, J., Gaughenbaugh, A., Renshaw, A., Wright, J., Seeber, R., Barnes, R., Florjanczyk, A., Horzempa, J., 2017. The potential for flower nectar to allow mosquito to mosquito transmission of *Francisella tularensis*. PLoS One 12, e0175157. doi:10.1371/journal.pone.0175157

Kerr, P.J., Ghedin, E., DePasse, J. V., Fitch, A., Cattadori, I.M., Hudson, P.J., Tscharke, D.C., Read, A.F., Holmes, E.C., 2012. Evolutionary history and attenuation of myxoma virus on two continents. PLoS Pathog. 8. doi:10.1371/journal.ppat.1002950

Kerr, P.J., Kitchen, A., Holmes, E.C., 2009. Origin and phylodynamics of rabbit hemorrhagic disease virus. J. Virol. 83, 12129–38. doi:10.1128/JVI.01523-09

Kiliç, S., Çelebi, B., Acar, B., Ataş, M., 2013. In vitro susceptibility of isolates of *Francisella tularensis* from Turkey. Scand. J. Infect. Dis. 45, 337–341. doi:10.3109/00365548.2012.751125

Kinnear, M., Linde, C.C., 2010. Capsid gene divergence in rabbit hemorrhagic disease virus. J. Gen. Virol. 91, 174–181. doi:10.1099/vir.0.014076-0

Kimura T, Mitsui I, Okada Y, Furuya T, Ochiai K, Umemura T, Itakura C., 2001. Distribution of rabbit haemorrhagic disease virus RNA in experimentally infected rabbits. J Comp Pathol, 124:134-14

Kishino, H., Thorne, J.L., Bruno, W.J., 2001. Performance of a divergence time estimation method under a probabilistic model of rate evolution. Mol. Biol. Evol. 18, 352–61. doi:10.1093/oxfordjournals.molbev.a003811

Kitchen, A., Shackelton, L.A., Holmes, E.C., 2011. Family level phylogenies reveal modes of macroevolution in RNA viruses. Proc. Natl. Acad. Sci. U. S. A. 108, 238–43. doi:10.1073/pnas.1011090108

Kokko, H., Lindström, J., 1998. Seasonal density dependence, timing of mortality, and sustainable harvesting. Ecological Modelling 110, 293–304

Kong S., 2015. On the use of Median-Joining networks: a philosophical and empirical evaluation of its suitability in evolutionary biology. MSc thesis. Department of Ecology and Evolutionary Biology. University of Toronto

König, M., Thiel, H.J., Meyers, G., 1998. Detection of viral proteins after infection of cultured hepatocytes with rabbit hemorrhagic disease virus. J. Virol. 72, 4492–7

Kovaliski, J., Sinclair, R., Mutze, G., Peacock, D., Strive, T., Abrantes, J., Esteves, P.J., Holmes, E.C., 2014. Molecular epidemiology of rabbit haemorrhagic disease virus in australia: When one became many. Mol. Ecol. 23, 408–420. doi:10.1111/mec.12596

Kovaliski, J., 1998. Monitoring the spread of rabbit hemorrhagic disease virus as a new biological agent for control of wild European rabbits in Australia. J. Wildl. Dis. 34, 421–428. doi:10.7589/0090-3558-34.3.421

Kreizinger, Z., Erdélyi, K., Felde, O., Fabbi, M., Sulyok, K.M., Magyar, T., Gyuranecz, M., 2016. Comparison of virulence of *Francisella tularensis* ssp. *holarctica* genotypes B.12 and B.FTNF002-00. BMC Vet. Res. 13, 46. doi:10.1186/s12917-017-0968-9

Kreizinger, Z., Hornok, S., Dán, A., Hresko, S., Makrai, L., Magyar, T., Bhide, M., Erdélyi, K., Hofmann-Lehmann, R., Gyuranecz, M., 2013a. Prevalence of *Francisella tularensis* and *Francisella*-like endosymbionts in the tick population of Hungary and the genetic variability of *Francisella*-like agents. Vector Borne Zoonotic Dis. 13, 160–3. doi:10.1089/vbz.2012.1065

Kreizinger, Z., Makrai, L., Helyes, G., Magyar, T., Erdélyi, K., Gyuranecz, M., 2013b. Antimicrobial susceptibility of *Francisella tularensis* subsp. *holarctica* strains from Hungary, Central Europe. J. Antimicrob. Chemother. 68, 370–373. doi:10.1093/jac/dks399

Kugeler, K.J., Mead, P.S., Janusz, A.M., Staples, J.E., Kubota, K.A., Chalcraft, L.G., Petersen, J.M., 2009. Molecular Epidemiology of *Francisella tularensis* in the United States. Clin. Infect. Dis. 48, 863–870. doi:10.1086/597261

Kugeler, K.J., Gurfield, N., Creek, J.G., Mahoney, K.S., Versage, J.L., Petersen, J.M., 2005. Discrimination between *Francisella tularensis* and *Francisella*-like endosymbionts when screening ticks by PCR. Appl. Environ. Microbiol. 71, 7594–7. doi:10.1128/AEM.71.11.7594-7597.2005

Kumar, S., Sunagar, R., Pham, G., Franz, B.J., Rosa, S.J., Hazlett, K.R.O., Gosselin, E.J., 2017. Differential cultivation of *Francisella tularensis* induces changes in the immune response to and protective efficacy of whole cell-based inactivated vaccines. Front. Immunol. 7, 677. doi:10.3389/fimmu.2016.00677

Kuroda, M., Sekizuka, T., Shinya, F., Takeuchi, F., Kanno, T., Sata, T., Asano, S., 2012. Detection of a possible bioterrorism agent, *Francisella* sp., in a clinical specimen by use of Next-Generation Direct DNA Sequencing. J. Clin. Microbiol. 50, 1810–1812. doi:10.1128/JCM.06715-11

Lai, X.-H., Zhao, L.-F., Chen, X.-M., Ren, Y., 2016. Rapid identification and characterization of *Francisella* by molecular biology and other techniques. Open Microbiol. J. 10, 64–77. doi:10.2174/1874285801610010064

Lalzar, I., Friedmann, Y., Gottlieb, Y., 2014. Tissue tropism and vertical transmission of *Coxiella* in *Rhipicephalus sanguineus* and *Rhipicephalus turanicus* ticks. Environ. Microbiol. 16, 3657–3668. doi:10.1111/1462-2920.12455

Larsson, P., Elfsmark, D., Svensson, K., Wikström, P., Forsman, M., Brettin, T., Keim, P., Johansson, A., 2009. Molecular evolutionary consequences of niche restriction in *Francisella tularensis*, a facultative intracellular pathogen. PLoS Pathog. 5, e1000472. doi:10.1371/journal.ppat.1000472

Larsson, P., Oyston, P.C.F., Chain, P., Chu, M.C., Duffield, M., Fuxelius, H.-H., Garcia, E., Hälltorp, G., Johansson, D., Isherwood, K.E., Karp, P.D., Larsson, E., Liu, Y., Michell, S., Prior, J., Prior, R., Malfatti, S., Sjöstedt, A., Svensson, K., Thompson, N., Vergez, L., Wagg, J.K., Wren, B.W., Lindler, L.E., Andersson,

S.G.E., Forsman, M., Titball, R.W., 2005. The complete genome sequence of *Francisella tularensis*, the causative agent of tularemia. Nat. Genet. 37, 153–9. doi:10.1038/ng1499

Laurent, S., Kut, E., Remy-Delaunay, S., Rasschaert, D., 2002. Folding of the rabbit hemorrhagic disease virus capsid protein and delineation of N-terminal domains dispensable for assembly. Arch. Virol. 147, 1559–1571. doi:10.1007/s00705-002-0825-3

Laurent, S., Vautherot, J.F., Madelaine, M.F., Le Gall, G., Rasschaert, D., 1994. Recombinant rabbit hemorrhagic disease virus capsid protein expressed in baculovirus self-assembles into virus-like particles and induces protection. J. Virol. 68, 6794–8

Lavazza, A., Capucci, L., 2008. How Many Caliciviruses are there in Rabbits? A Review on RHDV and Correlated Viruses, in: Lagomorph Biology. Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 263–278. doi:10.1007/978-3-540-72446-9_18

Lavazza, A., Cavadini, P., Barbieri, I., Tizzani, P., Pinheiro, A., Abrantes, J., Esteves, P.J., Grilli, G., Gioia, E., Zanoni, M., Meneguz, P., Guitton, J.-S., Marchandeau, S., Chiari, M., Capucci, L., 2015a. Field and experimental data indicate that the eastern cottontail (*Sylvilagus floridanus*) is susceptible to infection with European brown hare syndrome (EBHS) virus and not with rabbit haemorrhagic disease (RHD) virus. Vet. Res. 46, 13. doi:10.1186/s13567-015-0149-4

Lavazza, A., Tittarelli, C., Cerioli, M., 2015b. The use of convalescent sera in immune-electron microscopy to detect non-suspected/new viral agents. Viruses 7, 2683–2703. doi:10.3390/v7052683

Lebas,F,Coudert,P.,deRochambeau,H.The´bault,R.G.,1997.Therabbit;husbandry,healthandproduction.FAO AnimalProductionandHealthSeries,no.21.http://www.fao.org/docrep/t1690E/t1690e00. htm

Le Gall-Reculé, G., Lavazza, A., Marchandeau, S., Bertagnoli, S., Zwingelstein, F., Cavadini, P., Martinelli, N., Lombardi, G., Guérin, J.-L., Lemaitre, E., Decors, A., Boucher, S., Le Normand, B., Capucci, L., 2013. Emergence of a new lagovirus related to Rabbit Haemorrhagic Disease Virus. Vet. Res. 44, 81. doi:10.1186/1297-9716-44-81

Le Gall-Reculé, G., Zwingelstein, F., Boucher, S., Le Normand, B., Plassiart, G., Portejoie, Y., Decors, A., Bertagnoli, S., Guérin, J.-L., Marchandeau, S., 2011a. Detection of a new variant of rabbit haemorrhagic disease virus in France. Vet. Rec. 168, 137–8. doi:10.1136/vr.d697

Le Gall-Reculé, G., Zwingelstein, F., Fages, M.-P., Bertagnoli, S., Gelfi, J., Aubineau, J., Roobrouck, A., Botti, G., Lavazza, A., Marchandeau, S., 2011b. Characterisation of a non-pathogenic and non-protective infectious rabbit lagovirus related to RHDV. Virology 410, 395–402. doi:10.1016/j.virol.2010.12.001

Le Gall-Reculé, G., Zwingelstein, F., Laurent, S., de Boisséson, C., Portejoie, Y., Rasschaert, D., 2003. Phylogenetic analysis of rabbit haemorrhagic disease virus in France between 1993 and 2000, and the characterisation of RHDV antigenic variants. Arch. Virol. 148, 65–81. doi:10.1007/s00705-002-0908-1

Le Gall-Reculé G1, Zwingelstein F, Portejoie Y, Le Gall G. Immunocapture-RT-PCR assay for detection and molecular epidemiology studies of Rabbit Haemorrhagic Disease and European Brown Hare Syndrome viruses. J Virol Methods. 2001 Sep;97(1-2):49-57

Le Gall, G., Arnauld, C., Boilletot, E., Morisse, J.P., Rasschaert, D., 1998. Molecular epidemiology of rabbit

haemorrhagic disease virus outbreaks in France during 1988 to 1995. J. Gen. Virol. 79 (Pt 1), 11–6. doi:10.1099/0022-1317-79-1-11

Leighton, F.A., Artois, M., Capucci, L., Gavier-Widén, D., Morisse, J.-P., 1995. Antibody response to Rabbit Viral Hemorrhagic Disease Virus in Red Foxes (*Vulpes vulpes*) consuming livers of infected rabbits (*Oryctolagus cuniculus*). J. Wildl. Dis. 31, 541–544. doi:10.7589/0090-3558-31.4.541

Le Minor O., Beilvert F., Le Moullec T., Djadour D., Martineau J., 2013. Evaluation de l'efficacité d'un nouveau vaccin contre le virus variant de la maladie hémorragique virale du lapin (VHD). 15èmes Journées de la Recherche Cunicole, 19-20 novembre, Le Mans, France

Lenghaus C., Westbury H., Collins B., Ratnamohan M., Morrisy C., 1994. Overview of the RHD project in Australia. In Rabbit haemorrhagic disease: issues for assessment for biological control (R.K. Munro and R.T. Williams, eds) Australian Government Printing Service, Canberra, 104-129

Leisner V., 1983. As diferentes fases do Neolítico em Portugal. Arqueologia7, 7-15

Leland, D.S., Ginocchio, C.C., 2007. Role of cell culture for virus detection in the age of technology. Clin. Microbiol. Rev. 20, 49–78. doi:10.1128/CMR.00002-06

Lentacker A., 1986. Preliminary results of the fauna of Cabeço de Amoreira and Cabeço de Arruda (Muge, Portugal). Trabalhos de Antropologia e Etnologia26, 9-26.

Lester Rothfeldt, L.K., Jacobs, R.F., Wheeler, J.G., Weinstein, S., Haselow, D.T., 2017. Variation in Tularemia Clinical Manifestations—Arkansas, 2009–2013. Open Forum Infect. Dis. 4, ofx027. doi:10.1093/ofid/ofx027

Leuthold, M.M., Dalton, K.P., Hansman, G.S., 2015. Structural analysis of a rabbit hemorrhagic disease virus binding to histo-blood group antigens. J. Virol. 89, 2378–87. doi:10.1128/JVI.02832-14

Li J.N., 1990. Studies on interferon anti-RHDV activities in rabbits. J. Anim. Quar., 3, 19-22

Lin X.C., 1987. A study on RHD and pasteurellosis bivalent oil-emulsion vaccines (*in Chinese*). Chin. J. Anim. Pltry infec. Dis., 2, 9-1

Lindgren, H., Honn, M., Golovlev, I., Kadzhaev, K., Conlan, W., Sjöstedt, A., 2009. The 58-kilodalton major virulence factor of *Francisella tularensis* is required for efficient utilization of iron. Infect. Immun. 77, 4429–4436. doi:10.1128/IAI.00702-09

Liu, J.N., Yu, Z.J., Liu, L.M., Li, N.X., Wang, R.-R., Zhang, C.M., Liu, J.Z., 2016. Identification, distribution and population dynamics of *Francisella*-like endosymbiont in *Haemaphysalis doenitzi* (Acari: Ixodidae). Sci. Rep. 6, 35178. doi:10.1038/srep35178

Liu W., Dang R., Wang X., 2015. Development of a SYBR-based real-time PCR to detect rabbit hemorrhagic disease virus (RHDV) and analyze its tissue distribution in experimentally infected rabbits. Virol Sin. Jun;30(3):228-30. doi: 10.1007/s12250-015-3560-0

Liu, L., Li, L., Liu, J., Hu, Y., Liu, Z., Guo, L., Liu, J., 2013. Coinfection of *Dermacentor silvarum* olenev (acari: ixodidae) by *Coxiella*-Like, *Arsenophonus*-like, and *Rickettsia*-like symbionts. Appl. Environ. Microbiol. 79, 2450–4. doi:10.1128/AEM.03575-12

Liu J, Kerr PJ, Wright JD, Strive T., 2012. Serological assays to discriminate rabbit haemorrhagic disease virus from Australian non-pathogenic rabbit calicivirus. Vet Microbiol 157:345–354

Liu, G., Ni, Z., Yun, T., Yu, B., Chen, L., Zhao, W., Hua, J., Chen, J., 2008. A DNA-launched reverse genetics system for rabbit hemorrhagic disease virus reveals that the VP2 protein is not essential for virus infectivity. J. Gen. Virol. 89, 3080–5. doi:10.1099/vir.0.2008/003525-0

Liu, G., Zhang, Y., Ni, Z., Yun, T., Sheng, Z., Liang, H., Hua, J., Li, S., Du, Q., Chen, J., 2006. Recovery of infectious rabbit hemorrhagic disease virus from rabbits after direct inoculation with in vitro-transcribed RNA. J. Virol. 80, 6597–602. doi:10.1128/JVI.02078-05

Liu S.J., Xue H.P., Pu B.Q., Qian N.H., 1984. A new viral disease of rabbits. Animal Husbandry Vet Med.16, 253-255

Lobo, F.P., Mota, B.E.F., Pena, S.D.J., Azevedo, V., Macedo, A.M., Tauch, A., Machado, C.R., Franco, G.R., 2009. Virus-host coevolution: Common patterns of nucleotide motif usage in *Flaviviridae* and their hosts. PLoS One 4. doi:10.1371/journal.pone.0006282

Lopes de Carvalho, I., Toledo, A., Carvalho, C.L., Barandika, J.F., Respicio-Kingry, L.B., Garcia-Amil, C., García-Pérez, A.L., Olmeda, A.S., Zé-Zé, L., Petersen, J.M., Anda, P., Núncio, M.S., Escudero, R., 2016. *Francisella species* in ticks and animals, Iberian Peninsula. Ticks Tick. Borne. Dis. 7, 159–65. doi:10.1016/j.ttbdis.2015.10.009

Lopes de Carvalho, I., Zé-Zé, L., Alves, A.S., Pardal, S., Lopes, R.J., Mendes, L., Núncio, M.S., 2012. *Borrelia garinii* and *Francisella tularensis* subsp. *holarctica* detected in migratory shorebirds in Portugal. Eur. J. Wildl. Res. 58, 857–861. doi:10.1007/s10344-012-0617-3

Lopes de Carvalho I., Escudero R., García-Amil C., Falcão H., Anda P., Núncio M. S., 2007. *Francisella tularensis*, Portugal Emerg Infect Dis. 2007 Apr; 13(4): 666–667. doi: 10.3201/eid1304.060714

Lopes, A.M., Correia, J., Abrantes, J., Melo, P., Ramada, M., Magalhães, M.J., Alves, P.C., Esteves, P.J., 2015a. Is the new variant RHDV replacing genogroup 1 in Portuguese wild rabbit populations? Viruses 7, 27–36. doi:10.3390/v7010027

Lopes, A.M., Dalton, K.P., Magalhaes, M.J., Parra, F., Esteves, P.J., Holmes, E.C., Abrantes, J., 2015b. Full genomic analysis of new variant rabbit hemorrhagic disease virus revealed multiple recombination events. J. Gen. Virol. 96, 1309–1319. doi:10.1099/vir.0.000070

Lopes, A.M., Marques, S., Silva, E., Magalhães, M.J., Pinheiro, A., Alves, P.C., Le Pendu, J., Esteves, P.J., Thompson, G., Abrantes, J., 2014. Detection of RHDV strains in the Iberian hare (*Lepus granatensis*): earliest evidence of rabbit lagovirus cross-species infection. Vet. Res. 45, 94. doi:10.1186/s13567-014-0094-7

López, J. M., A. Hernández, F. J., Purroy Y., Robles J. L., 1996. Datos sobre la biología de la reproducción de la liebre Ibérica (*Lepus granatensis*) en agrosistemas cerealistas de la provincia de León (NW de España). Revista Florestal, 9(1): 49-60

Lopez-Martinez N., 1989. Revision sistematica y biostratigrafica de los lagomorphos (Mammalia) del neogeno y cuaternario de España. Memorias del Museo Paleontológico de la Universidad de Zaragoza, Diputación General de Aragón

Lopez-Martinez, N., 1980. Les lagomorphs (Mammalia) du ple´istoce`ne supe´rieur de Jaurens. Nouvelles Archives du Museum d'Histoire Naturelle de Lyon 18, 5–16

Lovisolo, O., Hull, R., Rösler, O., 2003. Coevolution of viruses with hosts and vectors and possible paleontology. Adv. Virus Res. 62, 325–379. doi:10.1016/S0065-3527(03)62006-3

Lowe, J., Gauger, P., Harmon, K., Zhang, J., Connor, J., Yeske, P., Loula, T., Levis, I., Dufresne, L., Main, R., 2014. Role of transportation in spread of porcine epidemic diarrhea virus infection, United States. Emerg. Infect. Dis. 20, 872–874. doi:10.3201/eid2005.131628

Luque, D., González, J.M., Gómez-Blanco, J., Marabini, R., Chichón, J., Mena, I., Angulo, I., Carrascosa, J.L., Verdaguer, N., Trus, B.L., Bárcena, J., Castón, J.R., 2012. Epitope insertion at the N-terminal molecular switch of the rabbit hemorrhagic disease virus T=3 capsid protein leads to larger T=4 capsids. J. Virol. 86, 6470–80. doi:10.1128/JVI.07050-11

Machín, A., Martín Alonso, J.M., Parra, F., 2001. Identification of the amino acid residue involved in rabbit hemorrhagic disease virus VPg uridylylation. J. Biol. Chem. 276, 27787–92. doi:10.1074/jbc.M100707200

Mahmood, T., Yang, P.C., 2012. Western blot: Technique, theory, and trouble shooting. N. Am. J. Med. Sci. 4, 429–434. doi:10.4103/1947-2714.100998

Maiden, M.C.J., van Rensburg, M.J.J., Bray, J.E., Earle, S.G., Ford, S.A., Jolley, K.A., McCarthy, N.D., 2013. MLST revisited: the gene-by-gene approach to bacterial genomics. Nat. Rev. Microbiol. 11, 728–736. doi:10.1038/nrmicro3093

Marboutin, E., Bray, Y., Péroux, R., Mauvy, B., Lartiges, A., 2003. Population dynamics in European hare: Breeding parameters and sustainable harvest rates. J. Appl. Ecol. 40, 580–591. doi:10.1046/j.1365-2664.2003.00813.x

Marboutin, E., Péroux Y. R., 1999. Some aspects of the spatial distribution of hares (*Lepus europaeus*) at night. Gibier Faune Sauvage, 16(2): 143-158

Marcato, P.S., Benazzi, C., Vecchi, G., Galeotti, M., Della Salda, L., Sarli, G., Lucidi, P., 1991. Clinical and pathological features of viral haemorrhagic disease of rabbits and the European brown hare syndrome. Rev. Sci. Tech. 10, 371–92

Marcato P.S., Benazzi C, Vecchi G., Della Salda L., Simoni P., Aiello P. & Tumino G. (1988). — L'epatite necrotica infettiva del coniglio. Profilo patogenetico di una nuova malattia emorragica. Riv. Coniglicoltura, 25 (9), 59-64

Marchandeau, S., Le Gall-Reculé, G., Bertagnoli, S., Aubineau, J., Botti, G., Lavazza, A., 2005. Serological evidence for a non-protective RHDV-like virus. Vet. Res. 36, 53–62. doi:10.1051/vetres:2004049

Marionneau, S., Ruvoën, N., Le Moullac-Vaidye, B., Clement, M., Cailleau-Thomas, A., Ruiz-Palacois, G., Huang, P., Jiang, X., Le Pendu, J., 2002. Norwalk virus binds to histo-blood group antigens present on gastroduodenal epithelial cells of secretor individuals. Gastroenterology 122, 1967–77

Marionneau, S., Cailleau-Thomas, A., Rocher, J., Le Moullac-Vaidye, B., Ruvoën, N., Clément, M., Le Pendu, J., 2001. ABH and Lewis histo-blood group antigens, a model for the meaning of oligosaccharide diversity in the face of a changing world. Biochimie. doi:10.1016/S0300-9084(01)01321-9

Marques, R.M., Teixeira, L., Aguas, A.P., Ribeiro, J.C., Costa-e-Silva, A., Ferreira, P.G., 2014.

Immunosuppression abrogates resistance of young rabbits to Rabbit Haemorrhagic Disease (RHD). Vet. Res. 45, 14. doi:10.1186/1297-9716-45-14

Marques, R.M., Costa-E-Silva, A., Águas, A.P., Teixeira, L., Ferreira, P.G., 2012. Early inflammatory response of young rabbits attending natural resistance to calicivirus (RHDV) infection. Vet. Immunol. Immunopathol. 150, 181–188. doi:10.1016/j.vetimm.2012.09.038

Marques, R.M., Costa-E-Silva, A., Águas, A.P., Teixeira, L., Ferreira, P.G., 2010. Early acute depletion of lymphocytes in calicivirus-infected adult rabbits. Vet. Res. Commun. 34, 659–668. doi:10.1007/s11259-010-9437-7

Marshall, I.D., Fenner, F., 1958. Studies in the epidemiology of infectious myxomatosis of rabbits: V. Changes in the innate resistance of Australian Wild rabbits exposed to myxomatosis. J. Hyg. (Lond). 56, 288. doi:10.1017/S0022172400037773

Martín-Alonso, A., Martin-Carrillo, N., Garcia-Livia, K., Valladares, B., Foronda, P., 2016. Emerging rabbit haemorrhagic disease virus 2 (RHDV2) at the gates of the African continent. Infect. Genet. Evol. 44, 46–50. doi:10.1016/j.meegid.2016.06.034

Martín-Alonso, J.M., Castañón, S., Alonso, P., Parra, F., Ordás, R., 2003. Oral Immunization using Tuber Extracts from Transgenic Potato Plants Expressing Rabbit Hemorrhagic Disease Virus Capsid Protein. Transgenic Res. 12, 127–130. doi:10.1023/A:1022112717331

Martín-Alonso, J.M., Casais, R., Boga, J.A., Parra, F., 1996. Processing of rabbit hemorrhagic disease virus polyprotein. J. Virol. 70, 1261–5

Martínez-Torrecuadrada, J.L., Cortés, E., Vela, C., Langeveld, J.P., Meloen, R.H., Dalsgaard, K., Hamilton, W.D., Casal, J.I., 1998. Antigenic structure of the capsid protein of rabbit haemorrhagic disease virus. J. Gen. Virol. 79 (Pt 8), 1901–9. doi:10.1099/0022-1317-79-8-1901

Martins, H., Milne, J.A., Rego, F., 2002. Seasonal and spatial variation in the diet of the wild rabbit (*Oryctolagus cuniculus* L.) in Portugal. J. Zool. 258, S0952836902001541. doi:10.1017/S0952836902001541

Martins, A.M.F., 1993. The Azores – Westernmost Europe: where evolution can be caught red-handed. Boletim do Museu Municipal do Funchal (Suppl. 2), 181–198

Matiz, K., Ursu, K., Kecskeméti, S., Bajmócy, E., Kiss, I., 2006. Phylogenetic analysis of rabbit haemorrhagic disease virus (RHDV) strains isolated between 1988 and 2003 in eastern Hungary. Arch. Virol. 151, 1659–1666. doi:10.1007/s00705-006-0730-2

Matthaei, M., Kerr, P.J., Read, A.J., Hick, P., Haboury, S., Wright, J.D., Strive, T., 2014. Comparative quantitative monitoring of rabbit haemorrhagic disease viruses in rabbit kittens. Virol. J. 11, 109. doi:10.1186/1743-422X-11-109

Matthee, C.A., van Vuuren, B.J., Bell, D., Robinson, T.J., 2004. A molecular supermatrix of the rabbits and hares (*Leporidae*) allows for the identification of five intercontinental exchanges during the Miocene. Syst. Biol. 53, 433–47

Maurin, M., Gyuranecz, M., 2016. Tularaemia: clinical aspects in Europe. Lancet Infect. Dis. 16, 113–124. doi:10.1016/S1473-3099(15)00355-2

Maurin, M., Pelloux, I., Brion, J.P., Del Banõ, J.N., Picard, A., 2011. Human Tularemia in France, 2006-2010. Clin. Infect. Dis. 53, e133–e141. doi:10.1093/cid/cir612

McColl, K.A., Merchant, J.C., Hardy, J., Cooke, B.D., Robinson, A., Westbury, H.A., 2002a. Evidence for insect transmission of rabbit haemorrhagic disease virus. Epidemiol. Infect. 129, 655–63

McColl KA, Morrissy CJ, Collins BJ, Westbury HA. 2002b. Persistence of rabbit haemorrhagic disease virus in decomposing rabbit carcasses. Aust Vet J; 80: 298–299

McIntosh, M.T., Behan, S.C., Mohamed, F.M., Lu, Z., Moran, K.E., Burrage, T.G., Neilan, J.G., Ward, G.B., Botti, G., Capucci, L., Metwally, S.A., 2007. A pandemic strain of calicivirus threatens rabbit industries in the Americas. Virol. J. 4, 96. doi:10.1186/1743-422X-4-96

Mellmann, A., Harmsen, D., Cummings, C.A., Zentz, E.B., Leopold, S.R., Rico, A., Prior, K., Szczepanowski, R., Ji, Y., Zhang, W., McLaughlin, S.F., Henkhaus, J.K., Leopold, B., Bielaszewska, M., Prager, R., Brzoska, P.M., Moore, R.L., Guenther, S., Rothberg, J.M., Karch, H., 2011. Prospective Genomic Characterization of the German Enterohemorrhagic *Escherichia coli* O104:H4 Outbreak by Rapid Next Generation Sequencing Technology. PLoS One 6, e22751. doi:10.1371/journal.pone.0022751

Melo-Ferreira, J., Boursot, P., Randi, E., Kryukov, A., Suchentrunk, F., Ferrand, N., Alves, P.C., 2007. The rise and fall of the mountain hare (*Lepus timidus*) during Pleistocene glaciations: Expansion and retreat with hybridization in the Iberian Peninsula. Mol. Ecol. 16, 605–618. doi:10.1111/j.1365-294X.2006.03166.x

Merchán, T., Rocha, G., Alda, F., Silva, E., Thompson, G., de Trucios, S.H., Pagés, A., 2011. Detection of rabbit haemorrhagic disease virus (RHDV) in nonspecific vertebrate hosts sympatric to the European wild rabbit (*Oryctolagus cuniculus*). Infect. Genet. Evol. 11, 1469–74. doi:10.1016/j.meegid.2011.05.001

Metropolis, N., Rosenbluth, A.W., Rosenbluth, M.N., Teller, A.H., Teller, E., 1953. Equation of State by Fast Computing Machines. J. Chem. Phys. 21, 1087–1092. doi:10.1063/1.1699114

Meunier, E., Wallet, P., Dreier, R.F., Costanzo, S., Anton, L., Rühl, S., Dussurgey, S., Dick, M.S., Kistner, A., Rigard, M., Degrandi, D., Pfeffer, K., Yamamoto, M., Henry, T., Broz, P., 2015. Guanylate-binding proteins promote activation of the AIM2 inflammasome during infection with *Francisella novicida*. Nat. Immunol. 16, 476–84. doi:10.1038/ni.3119

Meyers, G., 2007. Characterization of the sequence element directing translation reinitiation in RNA of the calicivirus rabbit hemorrhagic disease virus. J. Virol. 81, 9623–9632. doi:10.1128/JVI.00771-07

Meyers, G., 2003. Translation of the minor capsid protein of a calicivirus is initiated by a novel termination-dependent reinitiation mechanism. J. Biol. Chem. 278, 34051–34060. doi:10.1074/jbc.M304874200

Meyers, G., Wirblich, C., Thiel, H.-J., Thumfart, J.O., 2000. Rabbit Hemorrhagic Disease Virus: Genome Organization and Polyprotein Processing of a Calicivirus Studied after Transient Expression of cDNA Constructs. Virology 276, 349–363. doi:10.1006/viro.2000.0545

Meyers, G., Wirblich, C., Thiel, H.J., 1991a. Genomic and subgenomic RNAs of rabbit hemorrhagic disease virus are both protein-linked and packaged into particles. Virology 184, 677–686.

doi:10.1016/0042-6822(91)90437-G

Meyers, G., Wirblich, C., Thiel, H.J., 1991b. Rabbit hemorrhagic disease virus--molecular cloning and nucleotide sequencing of a calicivirus genome. Virology 184, 664–76

Mikami, O., Park, J.H., Kimura, T., Ochiai, K., Itakura, C., 1999. Hepatic lesions in young rabbits experimentally infected with rabbit haemorrhagic disease virus. Res. Vet. Sci. 66, 237–42. doi:10.1053/rvsc.1998.0266

Miller, W. a, Koev, G., 2000. Synthesis of subgenomic RNAs by positive-strand RNA viruses. Virology 273, 1–8. doi:10.1006/viro.2000.0421

Miller-Podraza, H., Stenhagen, G., Larsson, T., Andersson, C., Karlsson, K.A., 1997. Screening for the presence of polyglycosylceramides in various tissues: Partial characterization of blood group-active complex glycosphingolipids of rabbit and dog small intestines. Glycoconj. J. 14, 231–239. doi:10.1023/A:1018545922728

Mills, L.S., Doak, D.F., Doak, D.F., 1993. The keystone-species concept in ecology and conservation. Bioscience 43, 219–224. doi:10.2307/1312122

Mims, C.A., 1989. The pathogenetic basis of viral tropism. Am. J. Pathol. 135, 447–55

Mikschofsky H., Hartmann A., Janczyk P., Keil G. M., König P., Schirrmeier H., Hammer M., Junghans H., Schmidt K., Schmidtke J., Souffrant W. B., Schwerin M., Broer I. 2011. Expression of the viral antigen vp60 in transgenic potatoes and its effect on the nutritional composition of tubers. Food and Nutrition Sciences, 2, 74-86 doi:10.4236/fns.2011.22010 Published Online April 2011 (http://www.scirp.org/journal/fns)

Mitro, S., Krauss, H., 1993. Rabbit hemorrhagic disease: a review with special reference to its epizootiology. Eur. J. Epidemiol. 9, 70–78. doi:10.1007/BF00463093

Mitchell-Jones, A.J., Amori, G., Bogdanowicz, W., Kryštufek, B., Reijnders, P.J.H., Spitzenberger, F., Stubbe, M., Thissen, J.B.M., Vohralik, V. and Zima, J., 1999. The Atlas of European Mammals. Academic Press, London, UK

Mohapatra, N.P., Soni, S., Reilly, T.J., Liu, J., Klose, K.E., Gunn, J.S., 2008. Combined deletion of four *Francisella novicida* acid phosphatases attenuates virulence and macrophage vacuolar escape. Infect. Immun. 76, 3690–9. doi:10.1128/IAI.00262-08

Moinet, M., Decors, A., Mendy, C., Faure, E., Durand, B., Madani, N., 2016. Spatio-temporal dynamics of tularemia in French wildlife: 2002–2013. Prev. Vet. Med. 130, 33–40. doi:10.1016/j.prevetmed.2016.05.015

Monnerot, M., Vigne, J., Biju-Duval, C., Casane, D., Callou, C., Hardy, C., Mougel, F., Soriguer, R., Dennebouy, N., Mounolou, J., 1994. Rabbit and man: genetic and historic approach. Genet. Sel. Evol. 26, S167. doi:10.1186/1297-9686-26-S1-S167

Morales, M., Bárcena, J., Ramírez, M.A., Boga, J.A., Parra, F., Torres, J.M., 2004. Synthesis in vitro of rabbit hemorrhagic disease virus subgenomic RNA by internal initiation on (-)sense genomic RNA: mapping of a subgenomic promoter. J. Biol. Chem. 279, 17013–8. doi:10.1074/jbc.M313674200

Moran, N.A., 2008. Symbiosis. Curr.Biol. 16, 866-871

Moreno, S., Villafuerte, R., 1995. Traditional management of scrubland for the conservation of rabbits *Oryctolagus cuniculus* and their predators in Doñana National Park, Spain. Biol. Conserv. 73, 81–85. doi:10.1016/0006-3207(95)90069-1

Moret BME, Nakhleh L, Warnow T, Linder CR, Tholse A, Padolina A, Sun J, Timme R. 2004. Phylogenetic networks: modeling, reconstructibility, and accuracy. IEEE ACM Trans Comput Biol Bioinform. 1: 13–23

Morisse, J.-P., Le Gall, G., Boilletot, E., 1991. Hepatitis of viral origin in Leporidae: introduction and aetiological hypotheses. Rev. sci. tech. Off. int. Epiz 10, 283–295

Morrison D.A., 2010. Using data-display networks for exploratory data analysis in phylogenetic studies. Mol Biol Evol. May;27(5):1044-57. doi: 10.1093/molbev/msp309. Epub 2009 Dec 24.

Mörner, T., 1992. The ecology of tularaemia. Rev. Sci. Tech. 11, 1123–30

Morris, B.J., Buse, H.Y., Adcock, N.J., Rice, E.W., 2017. A novel broth medium for enhanced growth of *Francisella tularensis*. Lett. Appl. Microbiol. 64, 394–400. doi:10.1111/lam.12725

Moss, S.R., Turner, S.L., Trout, R.C., White, P.J., Hudson, P.J., Desai, A., Armesto, M., Forrester, N.L., Gould, E.A., 2002. Molecular epidemiology of rabbit haemorrhagic disease virus. J. Gen. Virol. 83, 2461–2467. doi:10.1099/0022-1317-83-10-2461

Mount, D.W., 2008. Choosing a method for phylogenetic prediction. Cold Spring Harb. Protoc. 3, pdb.ip49. doi:10.1101/pdb.ip49

Müller, A., Freitas, J., Silva, E., Le Gall-Reculé, G., Zwingelstein, F., Abrantes, J., Esteves, P.J., Alves, P.C., van der Loo, W., Kolodziejek, J., Nowotny, N., Thompson, G., 2009. Evolution of rabbit haemorrhagic disease virus (RHDV) in the European rabbit (*Oryctolagus cuniculus*) from the Iberian Peninsula. Vet. Microbiol. 135, 368–73. doi:10.1016/j.vetmic.2008.09.057

Müller, W., Hotzel, H., Otto, P., Karger, A., Bettin, B., Bocklisch, H., Braune, S., Eskens, U., Hörmansdorfer, S., Konrad, R., Nesseler, A., Peters, M., Runge, M., Schmoock, G., Schwarz, B.-A., Sting, R., Myrtennäs, K., Karlsson, E., Forsman, M., Tomaso, H., 2013. German *Francisella tularensis* isolates from European brown hares (*Lepus europaeus*) reveal genetic and phenotypic diversity. BMC Microbiol. 13, 61. doi:10.1186/1471-2180-13-61

Myers, K., Parer I., Wood D., Cooke B. D., 1994. The rabbit in Australia. Pages 108–157 in H. V. Thompson and C. M. King, editors. The European rabbit. The history and biology of a successful colonizer. Oxford University Press, New York

Nagesha, H.S., Wang, L.F., Hyatt, A.D., Morrissy, C.J., Lenghaus, C., Westbury, H.A., 1995. Self-assembly, antigenicity, and immunogenicity of the rabbit haemorrhagic disease virus (Czechoslovakian strain V-351) capsid protein expressed in baculovirus. Arch. Virol. 140, 1095–1108. doi:10.1007/BF01315418

Neimanis, A.S., Ahola, H., Zohari, S., Larsson Pettersson, U., Bröjer, C., Capucci, L., Gavier-Widén, D., 2017. Arrival of rabbit haemorrhagic disease virus 2 to northern Europe: Emergence and outbreaks in wild and domestic rabbits (*Oryctolagus cuniculus*) in Sweden. Transbound. Emerg. Dis. doi:10.1111/tbed.12650

Nhu Nguyen, T.M., Ilef, D., Jarraud, S., Rouil, L., Campese, C., Che, D., Haeghebaert, S., Ganiayre, F.,

Marcel, F., Etienne, J., Desenclos, J., 2006. A Community‐Wide Outbreak of Legionnaires Disease Linked to Industrial Cooling Towers—How Far Can Contaminated Aerosols Spread? J. Infect. Dis. 193, 102–111. doi:10.1086/498575

Niebylski, M.L., Peacock, M.G., Fischer, E.R., Porcella, S.F., Schwan, T.G., 1997. Characterization of an endosymbiont infecting wood ticks, *Dermacentor andersoni*, as a member of the genus *Francisella*. Appl. Environ. Microbiol. 63, 3933–40

Niedzwiedzka-Rystwej, P., Hukowska-Szematowicz, B., Dzialo, J., Tokarz-Deptula, B. and Deptula, W., 2013. Real time PCR detection of rabbit haemorrhagic disease virus in rabbits infected with different European strains of RHDV. Pol J Vet Sci. 16, 39-43

Nikoh, N., Hosokawa, T., Moriyama, M., Oshima, K., Hattori, M., Fukatsu, T., 2014. Evolutionary origin of insect-*Wolbachia* nutritional mutualism. Proc. Natl. Acad. Sci. U. S. A. 111, 10257–62. doi:10.1073/pnas.1409284111

Noda, H., Munderloh, U.G., Kurtti, T.J., 1997. Endosymbionts of ticks and their relationship to *Wolbachia* spp. and tick-borne pathogens of humans and animals. Appl. Environ. Microbiol. 63, 3926–32.

Norkin L.C., 1995. Virus receptors: implications for pathogenesis and the design of antiviral agents.

Clin Microbiol Rev. Apr; 8(2): 293–315

Notomi T., Okayama H., Masubuchi H., Yonekawa T., Watanabe K., Amino N., Hase T., 2000. Loop-mediated isothermal amplification of DNA. Nucleic Acids Res. Jun 15;28(12):E63

Nowotny, N., Bascuñana, C.R., Ballagi-Pordány, A., Gavier-Widén, D., Uhlén, M., Belák, S., 1997. Phylogenetic analysis of rabbit haemorrhagic disease and European brown hare syndrome viruses by comparison of sequences from the capsid protein gene. Arch. Virol. 142, 657–73

Nowotny, N., Steineck T., Tataruch F., Schilcher F., Weissenbock H., 1991. European brown hare syndrome (EBHS)-Experimentelle Untersuchungen. Wien. Tierarztl. Monschr. 78: 370-378

Nowotny, N., Fuchs A., Schilcher F., and Loupal G., 1990. Zum Auftreten der rabbit haemorrhagic disease (RHD) in Osterreich. I. Pathomorphologische und virologische Untersuchungen. Wien. Tierarztl. Monschr. 77:19-23

Núncio, M.S., 2002. Contribuição para o estudo de borrélias e borreliose de Lyme em Portugal. Report for PhD in Biology. Science Faculty, Universidade de Lisboa

Nyström, K., Le Moullac-Vaidye, B., Ruvoën-Clouet, N., Le Pendu, J., 2012. Shared human/rabbit ligands for rabbit hemorrhagic disease virus. Emerg. Infect. Dis. 18, 518–519. doi:10.3201/eid1803.111402

Nyström, K., Le Gall-Reculé, G., Grassi, P., Abrantes, J., Ruvoën-Clouet, N., Le Moullac-Vaidye, B., Lopes, A.M., Esteves, P.J., Strive, T., Marchandeau, S., Dell, A., Haslam, S.M., Le Pendu, J., 2011. Histo-blood group antigens act as attachment factors of rabbit hemorrhagic disease virus infection in a virus strain-dependent manner. PLoS Pathog. 7, e1002188. doi:10.1371/journal.ppat.1002188

O 'hara, P., 2006. The illegal introduction of rabbit haemorrhagic disease virus in New Zealand. Rev. sci. tech. Off. int. Epiz 25, 119–123

Oh, H., Kim, C.Y., Kim, C.H., Hur, G.H., Park, J.H., 2016. A synthetic Tul4 and FopA peptide cocktail of *Francisella tularensis* induces humoral and cell-mediated immune responses in mice. J. Microbiol.

Biotechnol. 26, 1613–1619. doi:10.4014/jmb.1602.02022

Ohlinger, V.F., Haas, B., Meyers, G., Weiland, F., Thiel, H.J., 1990. Identification and characterization of the virus causing rabbit hemorrhagic disease. J. Virol. 64, 3331–3336.

Ohlinger, V.F., Haas, B., Thiel, H.J., 1993. Rabbit hemorrhagic disease (RHD): Characterization of the causative calicivirus. Vet. Res. BioMed Central. 24 (2), pp.103-116

OIE (World Organisation for Animal Health) technical disease cards 2015.http://www.oie.int/animal-health-in-the-world/technical-disease-cards/

OIE (World Organisation for Animal Health) Technical disease cards. 2016. Rabbit Haemorrhagic Disese. Aetology, Epidemiology, Diagnosis, Prevention and Control References

OIE Terrestrial manual. Chapter 2.6.2. Rabbit haemorrhagic disease

Olivo P.D., 1996. Transgenic cell lines for detection of animal viruses. Clin Microbiol Rev. Jul; 9(3): 321–334

Olsufjev, N.G., Meshchekyakova, D.I.S., La Remiu La Hora Tory, T., 1983. Subspecific Taxonomy of *Francisella tularensis* McCoy and Chapin 1912t 872–874

Origgi, F.C., Frey, J., Pilo, P., 2014. Characterisation of a new group of *Francisella tularensis* subsp. *holarctica* in Switzerland with altered antimicrobial susceptibilities, 1996 to 2013. Euro Surveill. 19

Origgi, F.C., Pilo, P., 2016. *Francisella tularensis* clades B.FTN002-00 and B.13 are associated with distinct pathology in the European Brown Hare (*Lepus europaeus*). Vet. Pathol. 53, 1220–1232. doi:10.1177/0300985816629718

Oriol, R., Mollicone, R., Coullin, P., Dalix, A.M., Candelier, J.J., 1992. Genetic regulation of the expression of ABH and Lewis antigens in tissues. APMIS 100, 28–38. doi:10.1111/j.1600-0463.1992.tb05421.x

Ozanic, M., Marecic, V., Lindgren, M., Sjöstedt, A., Santic, M., 2016. Phenotypic characterization of the *Francisella tularensis* ΔpdpC and ΔiglG mutants. Microbes Infect. 18, 768–776. doi:10.1016/j.micinf.2016.07.006

Page, R.D.M., 2003. Tangled Trees: Phylogeny, Cospeciation, and Coevolution. The University of Chicago Press, Chicago. 350 pp.

Pages M.V., 1980. Essai de reconstitution de l'histoire du lapin de garenne en Europe. Bull Mens Off Natl Chasse, 13-21

Paixão R., Godinho S., Santos P., 2009. Is the Nature 2000 Network associated with small-game bag results? Eur J Wildl Res 55:553–559

Palacios, F., 1976. Descripción de una nueva especie de liebre (*Lepus castroviejoi*), endémica de la Cordillera Cantábria. Doñana, Acta Vertebrata, 3(2): 205-223

Palacios, F., Fernandez, J., 1992. A new subspecies of hare from Majorca (Balearic Islands). Mammalia 56: 71-85

Park JH, Lee YS, Itakura C: Pathogenesis of acute necrotic hepatitis in rabbit hemorrhagic disease. Lab Anim Sci 1995, 45:445-449

Park N.Y., Chong C.Y., Kim J.H., Cho S.M., Cha Y.H., Jung B.T., Kim D.S., Yoon J.B., 1987. An outbreak of viral haemorrhagic pneumonia (tentative name) of rabbits in Korea. J Korean Vet Med Assoc, 23:603-

610

Parkes, J.P., Heyward, R.P., Henning, J., Motha, M.X.J., 2004. Antibody responses to rabbit haemorrhagic disease virus in predators, scavengers, and hares in New Zealand during epidemics in sympatric rabbit populations. N. Z. Vet. J. 52, 85–9. doi:10.1080/00480169.2004.36410

Parkes, J.P., Glentworth, B., Sullivan, G., 2008. Changes in immunity to rabbit haemorrhagic disease virus, and in abundance and rates of increase of wild rabbits in Mackenzie Basin, New Zealand. Wildl. Res. 35, 775. doi:10.1071/WR08008

Parra, F., Boga, J.A., Marin, M.S., Casais, R., 1993. The amino terminal sequence of VP60 from rabbit hemorrhagic disease virus supports its putative subgenomic origin. Virus Res. 27, 219–28

Parra, F., Prieto, M., 1990. Purification and characterization of a calicivirus as the causative agent of a lethal hemorrhagic disease in rabbits. J. Virol. 64, 4013–4015

Pavlovich, N. V, Mishan'kin, B.N., 1987. Transparent nutrient medium for culturing *Francisella tularensis*. Antibiot. i meditsinskaia biotekhnologiia = Antibiot. Med. Biotechnol. 32, 133–7.

Pavlovsky, E. N., 1966. Natural nidality of transmissible diseases. Urbana Illinois: University of Illinois Press

Payne, L., Arneborn, M., Tegnell, A., Giesecke, J., 2005. Endemic Tularemia, Sweden, 2003. Emerg. Infect. Dis. 11, 1440–1442. doi:10.3201/eid1109.041189

Pechous, R.D., McCarthy, T.R., Mohapatra, N.P., Soni, S., Penoske, R.M., Salzman, N.H., Frank, D.W., Gunn, J.S., Zahrt, T.C., 2008. A *Francisella tularensis* SCHU S4 purine auxotroph is highly attenuated in mice but offers limited protection against homologous intranasal challenge. PLoS One 3, e2487. doi:10.1371/journal.pone.0002487

Pechous, R., Celli, J., Penoske, R., Hayes, S.F., Frank, D.W., Zahrt, T.C., 2006. Construction and characterization of an attenuated purine auxotroph in a *Francisella tularensis* live vaccine strain. Infect. Immun. 74, 4452–4461. doi:10.1128/IAI.00666-06

Pérez-Filgueira, D.M., Resino-Talaván, P., Cubillos, C., Angulo, I., Barderas, M.G., Barcena, J., Escribano, J.M., 2007. Development of a low-cost, insect larvae-derived recombinant subunit vaccine against RHDV. Virology 364, 422–430. doi:10.1016/j.virol.2007.03.016

Pérez-Suárez, G., Palacios, F., Boursot, P., 1994. Speciation and paraphyly in western Mediterranean hares (*Lepus castroviejoi*, *L. europaeus*, *L. granatensis*, and *L. capensis*) revealed by mitochondrial DNA phylogeny. Biochem. Genet. 32, 423–36

Perlman, S.J., Hunter, M.S., Zchori-Fein, E., 2006. The emerging diversity of *Rickettsia*. Proceedings. Biol. Sci. 273, 2097–106. doi:10.1098/rspb.2006.3541

Petersen, J.M., Carlson, J., Yockey, B., Pillai, S., Kuske, C., Garbalena, G., Pottumarthy, S., Chalcraft, L., 2009. Direct isolation of *Francisella* spp. from environmental samples. Lett. Appl. Microbiol. 48, 663–7. doi:10.1111/j.1472-765X.2009.02589.x

Petersen, J.M., Schriefer, M.E., 2005. Tularemia: Emergence/re-emergence. Vet. Res. doi:10.1051/vetres:2005006

Petersen, M.R., Deddens, J.A., 2008. A comparison of two methods for estimating prevalence ratios. BMC

Med Res Methodol 8, 9. doi:10.1186/1471-2288-8-9

Pikula J., Beklova M., Holesovska Z., Treml F., 2004. Prediction of possible distribution of tularemia in the Czech Republic. Vet Med-Czech, 49:61-64

Plana-Duran, J., Bastons, M., Rodriguez, M.J., Climent, I., Cortes, E., Vela, C., Casal, I., 1996. Oral immunization of rabbits with VP60 particles confers protection against rabbit hemorrhagic disease. Arch Virol 141, 1423–36. doi:10.1007/BF01718245

Ponchel F., Toomes C., Bransfield K., Leong F.T., Douglas S.H., Field S.L., Bell S.M., Combaret V., Puisieux A., Mighell A.J., Robinson P.A., Inglehearn C.F., Isaacs J.D., Markham A.F. 2003. Real-time PCR based on SYBR-Green I fluorescence: an alternative to the TaqMan assay for a relative quantification of gene rearrangements, gene amplifications and micro gene deletions. BMC Biotechnol. Oct 13;3:18.

Posada D., Crandall K.A., 1998. MODELTEST: Testing the model of DNA substitution. Bioinformatics 14(9): 817–818

Powell, D.A., Frelinger, J.A., 2017. Efficacy of resistance to Francisella imparted by ITY/NRAMP/SLC11A1 depends on route of infection. Front. Immunol. 8, 206. doi:10.3389/fimmu.2017.00206

Prieto J.M., Fernandez F., Alvarez V., Espi A., Garcia Marin J.F., Alvarez M., Martin J.M., Parra F., 2000. Immunohistochemical localisation of rabbit haemorrhagic disease virus VP-60 antigen in early infection of young and adult rabbits. Res Vet Sci, 68:181-187

Pu B.Q., Quian N.H., Cui S.J., 1985. Micro HA and HI tests for the detection of antibody titres to so-called "haemorrhagic pneumonia" in rabbits (in Chinese). Chin. J. vet. Med., 11 (10), 16-17

Puggioni, G., Cavadini, P., Maestrale, C., Scivoli, R., Botti, G., Ligios, C., Le Gall-Reculé, G., Lavazza, A., Capucci, L., 2013. The new French 2010 Rabbit Hemorrhagic Disease Virus causes an RHD-like disease in the Sardinian Cape hare (*Lepus capensis mediterraneus*). Vet. Res. 44, 96. doi:10.1186/1297-9716-44-96

Purroy, F. J., 2011. Liebre ibérica – *Lepus granatensis*. En: Enciclopedia Virtual de los Vertebrados Españoles. Salvador, A., Cassinello, J. (Eds.). Museo Nacional de Ciencias Naturales, Madrid. http://www.vertebradosibericos.org/

Qin, A., Scott, D.W., Thompson, J.A., Mann, B.J., 2009. Identification of an essential *Francisella tularensis* subsp. *tularensis* virulence factor. Infect. Immun. 77, 152–161. doi:10.1128/IAI.01113-08

Qin, A., Mann, B.J., 2006. Identification of transposon insertion mutants of *Francisella tularensis* tularensis strain SCHU S4 deficient in intracellular replication in the hepatic cell line HepG2. BMC Microbiol. 6, 69. doi:10.1186/1471-2180-6-69

Qu, P., Deng, X., Zhang, J., Chen, J., Zhang, J., Zhang, Q., Xiao, Y., Chen, S., 2009. Identification and characterization of the *Francisella* sp. strain 08HL01032 isolated in air condition systems. Wei Sheng Wu Xue Bao 49, 1003–10

Queney, G., Ferrand, N., Weiss, S., Mougel, F., Monnerot, M., 2001. Stationary Distributions of Microsatellite Loci Between Divergent Population Groups of the European Rabbit (*Oryctolagus cuniculus*). Mol. Biol. Evol. 18, 2169–2178. doi:10.1093/oxfordjournals.molbev.a003763

Quijada, S.G., Díaz, M.R., Ortega, J.L.Y., Martínez, I.C., Fernández, E.O., García, J.L., 2002. Tularemia: study

of 27 patients. Medicina Clınica 119: 455-457

Ramakrishnan, G., 2017. Iron and Virulence in *Francisella tularensis*. Front. Cell. Infect. Microbiol. 7, 107. doi:10.3389/fcimb.2017.00107

Ramakrishnan, G., Meeker, A., Dragulev, B., 2008. FslE is necessary for siderophore-mediated iron acquisition in *Francisella tularensis* SCHU S4. J. Bacteriol. 190, 5353–5361. doi:10.1128/JB.00181-08

Ramakrishnan, G., Sen, B., Johnson, R., 2012. Paralogous outer membrane proteins mediate uptake of different forms of iron and synergistically govern virulence in *Francisella tularensis tularensis*. J. Biol. Chem. 287, 25191–25202. doi:10.1074/jbc.M112.371856

Rambaut, A., 2000. Estimating the rate of molecular evolution: incorporating non-contemporaneous sequences into maximum likelihood phylogenies. Bioinformatics 16, 395–399. doi:10.1093/bioinformatics/16.4.395

Rambaut, A., Lam, T.T., Max Carvalho, L., Pybus, O.G., 2016. Exploring the temporal structure of heterochronous sequences using TempEst (formerly Path-O-Gen). Virus Evol. 2, vew007. doi:10.1093/ve/vew007

Rannala, B., Yang, Z.H., 1996. Probability distribution of molecular evolutionary trees: A new method of phylogenetic inference. J. Mol. Evol. 43, 304–311. doi:10.1007/BF02338839

Raoult, D., Fournier, P.-E., Abboud, P., Caron, F., 2002. First documented human *Rickettsia aeschlimannii* infection. Emerg. Infect. Dis. 8, 748–9. doi:10.3201/eid0807.010480

Richard, K., Mann, B.J., Qin, A., Barry, E.M., Ernst, R.K., Vogel, S.N., 2017. Monophosphoryl lipid a enhances efficacy of a *Francisella tularensis* LVS catanionic nanoparticle subunit vaccine against *F. tularensis* SCHU S4 challenge by augmenting both humoral and cellular immunity. Clin. Vaccine Immunol. 24, e00574–16. doi:10.1128/CVI.00574-16

Richard, K., Mann, B.J., Stocker, L., Barry, E.M., Qin, A., Cole, L.E., Hurley, M.T., Ernst, R.K., Michalek, S.M., Stein, D.C., DeShong, P., Vogel, S.N., 2014. Novel catanionic surfactant vesicle vaccines protect against *Francisella tularensis* LVS and confer significant partial protection against *F. tularensis* SCHU S4 strain. Clin. Vaccine Immunol. 21, 212–226. doi:10.1128/CVI.00738-13

Rigard, M., Bröms, J.E., Mosnier, A., Hologne, M., Martin, A., Lindgren, L., Punginelli, C., Lays, C., Walker, O., Charbit, A., Telouk, P., Conlan, W., Terradot, L., Sjöstedt, A., Henry, T., 2016. *Francisella tularensis* IglG Belongs to a Novel Family of PAAR-Like T6SS Proteins and Harbors a Unique N-terminal Extension Required for Virulence. PLOS Pathog. 12, e1005821. doi:10.1371/journal.ppat.1005821

Rijks, J.M., Kik, M., Koene, M.G., Engelsma, M.Y., van Tulden, P., Montizaan, M.G., Oomen, T., Spierenburg, M.A., Ijzer, J., van der Giessen, J.W., Gröne, A., Roest, H.J., 2013. Tularaemia in a brown hare (*Lepus europaeus*) in 2013: First case in the Netherlands in 60 years. Eurosurveillance 18. doi:10.2807/1560-7917.ES2013.18.49.20655

Roberts, L.M., Wehrly, T.D., Crane, D.D., Bosio, C.M., 2017. Expansion and retention of pulmonary CD4+ T cells after prime boost vaccination correlates with improved longevity and strength of immunity against tularemia. Vaccine 35, 2575–2581. doi:10.1016/j.vaccine.2017.03.064

Rodák L., Granatova M., Valicek L., Smid B., Vesely T., Nevorankova, Z., 1990a. - Monoclonal antibodies

to rabbit haemorrhagic disease virus and their use in the diagnosis of infection. J. gen. Virol., 71 (5), 2593-2598

Rodák, L., Smid, B., Valicek, L., Vesely, T., Stepanek, J., Hampl, J., Jurak, E., 1990b. Enzyme-linked Immunosorbent Assay of Antibodies to Rabbit Haemorrhagic Disease Virus and Determination of its Major Structural Proteins. J. Gen. Virol. 71, 1075–1080. doi:10.1099/0022-1317-71-5-1075

Rohmer, L., Brittnacher, M., Svensson, K., Buckley, D., Haugen, E., Zhou, Y., Chang, J., Levy, R., Hayden, H., Forsman, M., Olson, M., Johansson, A., Kaul, R., Miller, S.I., 2006. Potential source of *Francisella tularensis* live vaccine strain attenuation determined by genome comparison. Infect. Immun. 74, 6895–906. doi:10.1128/IAI.01006-06

Roingeard, P., 2008. Viral detection by electron microscopy: past, present and future. Biol. Cell 100, 491–501. doi:10.1042/BC20070173

Romanova, L. V., Mishan'kin, B. N., Pichurina, N. L., Vodop'ianov, S. O., Saiamov, S. R., 2000. Noncultivatable forms of *Francisella tularensis*. Zh Mikrobiol Epidemiol Immunobiol, 11–15.

Ros Bascunana, C., Nowotny, N. and Belak, S., 1997. Detection and differentiation of rabbit hemorrhagic disease and European brown hare syndrome viruses by amplification of VP60 genomic sequences from fresh and fixed tissue specimens. J Clin Microbiol. 1997 Oct;35(10):2492-5

Ruvoën-Clouet, N., Ganière, J.P., André-Fontaine, G., Blanchard, D., Le Pendu, J., 2000. Binding of rabbit hemorrhagic disease virus to antigens of the ABH histo-blood group family. J. Virol. 74, 11950–4.

Ruvoën-Clouet, N., Blanchard, D., Andre-Fontaine, G., Ganiere, J., 1995. Partial characterization of the human erythrocyte receptor for rabbit haemorrhagic disease virus. Inst. Pasteljrelsevier Res. Virol. Paris 146, 33–41

Saitou, N., Imanishi, T., 1989. Relative efficiencies of the Fitch-Margoliash, maximum-parsimony, maximum-likelihood, minimum-evolution, and neighbor-joining methods of phylogenetic tree construction in obtaining the correct tree. Mol. Biol. Evol 6, 514–525. doi:10.1093/oxfordjournals.molbev.a040572

Saitou N. and Nei M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol. Jul;4(4):406-25

Sanarelli, G., 1898. Das myxomatogene Virus. Beitrag zum Stadium der Krankheit-serreger ausserhalbdes Sichtbaren. Zbl. Bakt 23, 865-873

Sanderson, M.J., 2003. r8s: Inferring absolute rates of molecular evolution and divergence times in the absence of a molecular clock. Bioinformatics 19, 301–302. doi:10.1093/bioinformatics/19.2.301

Santic, M., Molmeret, M., Klose, K.E., Jones, S., Abu Kwaik, Y., 2005. The *Francisella tularensis* pathogenicity island protein IglC and its regulator MglA are essential for modulating phagosome biogenesis and subsequent bacterial escape into the cytoplasm. Cell. Microbiol. 7, 969–979. doi:10.1111/j.1462-5822.2005.00526.x

Schirrmeier, H., Reimann, I., Köllner, B., Granzow, H., 1999. Pathogenic, antigenic and molecular properties of rabbit haemorrhagic disease virus (RHDV) isolated from vaccinated rabbits: detection and characterization of antigenic variants. Arch. Virol. 144, 719–35

Schirrmeier H., Granzow H., Bergmann H., Schlüter H., 1990. Experimentelle Untersuchungen zur hämorrhagischen Septikämie der Kaninchen. Mh. VetMed., 45, 193-197

Schliep K.P., 2011. phangorn: phylogenetic analysis in R. Bioinformatics. Feb 15;27(4):592-3. doi: 10.1093/bioinformatics/btq706. Epub 2010 Dec 17

Schramlová, J., Arientová, S., Hulínská, D., 2010. The role of electron microscopy in the rapid diagnosis of viral infections — review. Folia Microbiol. (Praha). 55, 88–101. doi:10.1007/s12223-010-0015-8

Schulert, G.S., McCaffrey, R.L., Buchan, B.W., Lindemann, S.R., Hollenback, C., Jones, B.D., Allen, L.A.H., 2009. *Francisella tularensis* genes required for inhibition of the neutrophil respiratory burst and intramacrophage growth identified by random transposon mutagenesis of strain LVS. Infect. Immun. 77, 1324–1336. doi:10.1128/IAI.01318-08

Scicluna M.T., Gamba D., Vecchi G., Brivio R., Capucci L., 1990. Metodiche diagnostiche applicate alla malattia emorragica virale del coniglio (RVHD): 2° Analisi serologica. In 44° Convegno Nazionale Società Italiana delle Scienze Veterinarie, Stresa (Italy), 27-29 September (in press)

Scoles, G.A., 2004. Phylogenetic Analysis of the *Francisella* -like Endosymbionts of Dermacentor Ticks. J. Med. Entomol. 41, 277–286. doi:10.1603/0022-2585-41.3.277

Sen, B., Meeker, A., Ramakrishnan, G., 2010. The fslE homolog, FTL-0439 (fupA/B), mediates siderophore-dependent iron uptake in *Francisella tularensis* LVS. Infect. Immun. 78, 4276–4285. doi:10.1128/IAI.00503-10

Sharp, P.M., Simmonds, P., 2011. Evaluating the evidence for virus/host co-evolution. Curr. Opin. Virol. 1, 436–441. doi:10.1016/j.coviro.2011.10.018

Shien, J.H., Shieh, H.K., Lee, L.H., 2000. Experimental infections of rabbits with rabbit haemorrhagic disease virus monitored by polymerase chain reaction. Res. Vet. Sci. 68, 255–259. doi:10.1053/rvsc.1999.0372

Sibilia, M., Boniotti, M.B., Angoscini, P., Capucci, L., Rossi, C., 1995. Two independent pathways of expression lead to self-assembly of the rabbit hemorrhagic disease virus capsid protein. J. Virol. 69, 5812–5815

Simmonds, P., Karakasiliotis, I., Bailey, D., Chaudhry, Y., Evans, D.J., Goodfellow, I.G., 2008. Bioinformatic and functional analysis of RNA secondary structure elements among different genera of human and animal caliciviruses. Nucleic Acids Res. 36, 2530–46. doi:10.1093/nar/gkn096

Sjödin, A., Svensson, K., Öhrman, C., Ahlinder, J., Lindgren, P., Duodu, S., Johansson, A., Colquhoun, D.J., Larsson, P., Forsman, M., 2012. Genome characterisation of the genus *Francisella* reveals insight into similar evolutionary paths in pathogens of mammals and fish. BMC Genomics 13, 268. doi:10.1186/1471-2164-13-268

Sjöstedt, A., 2007. Tularemia: History, epidemiology, pathogen physiology, and clinical manifestations. Ann. N. Y. Acad. Sci. doi:10.1196/annals.1409.009

Smid B, Valícek L, Rodak L, Stepanek J, Jurak E., 1991. Rabbit haemorrhagic disease: an investigation of some properties of the virus and evaluation of an inactivated vaccine. Vet Microbiol. 26: 77–85.

Smid B, Valícek J, Stephanek J, Jurak E, Rodák L., 1989. Experimental transmission and electron

microscopic demonstration of the virus of haemorrhagic disease of rabbits in Czechoslovakia. J Vet Med; 36: 237– 240

Smith, D.J., Timonen, H.J., Jaffe, D.A., Griffin, D.W., Birmele, M.N., Perry, K.D., Ward, P.D., Roberts, M.S., 2013. Intercontinental dispersal of bacteria and archaea by transpacific winds. Appl. Environ. Microbiol. 79, 1134–1139. doi:10.1128/AEM.03029-12

Smith, A.T., Boyer, A.F., 2008. *Oryctolagus cuniculus*. The IUCN Red List of Threatened Species 2008: e.T41291A10415170. http://dx.doi.org/10.2305/IUCN.UK.2008.RLTS.T41291A10415170.en. Downloaded on 08 July 2017

Smith, A.T., Johnston, C.H., 2008. *Lepus granatensis*. The IUCN Red List of Threatened Species 2008: e.T41306A10437192. http://dx.doi.org/10.2305/IUCN.UK.2008.RLTS.T41306A10437192.en. Downloaded on 17 July 2017

Smith, G., 1997. An analysis of the form of density dependence in a simulation model of a seasonal breeder undergoing control. Ecol. Modell. 95, 181–189.

Smith, G., Trout, R., 1994. Using Leslie matrices to determine wild rabbit population growth and the potential for control. J. Appl. Ecol. Vol. 31. 2: 223-30

Snoy, P.J., 2010. Establishing Efficacy of Human Products Using Animals. Vet. Pathol. Online 47, 774 – 778. doi:10.1177/0300985810372506

Soriguer R.C., 1980. El conejo, *Oryctolagus cuniculus* (L), en Andalucía Occidental: Parámetros corporales y curva de crecimiento. Doñana, Acta Vertebr., 7: 83-90.

Sosnovtsev S.V., 2010. Proteolytic cleavage and viral proteins, Chapter 4. In: Hansman GS, Jiang XJ, Green KY (eds) Caliciviruses: molecular and cellular virology. Caister Academic Press, ISBN: 978-1-904455-63-9

Sosnovtsev, S. V., Belliot, G., Chang, K.O., Onwudiwe, O., Green, K.Y., 2005. Feline calicivirus VP2 is essential for the production of infectious virions. J. Virol. 79, 4012–24. doi:10.1128/JVI.79.7.4012-4024.2005

Sosnovtsev, S. V, Prikhod'ko, E.A., Belliot, G., Cohen, J.I., Green, K.Y., 2003. Feline calicivirus replication induces apoptosis in cultured cells. Virus Res. 94, 1–10

Sosnovtsev, S. V, Green, K.Y., 2000. Identification and genomic mapping of the ORF3 and VPg proteins in feline calicivirus virions. Virology 277, 193–203. doi:10.1006/viro.2000.0579

Souiri A., Zemzami M., Amzazi S., Ennaji M.M., 2016. Recent advances in virus-host coevolution and protective mechanisms against plant viruses. British Microbiology Research Journal 11(2): 1-18, , Article no.BMRJ.20439 ISSN: 2231-0886, NLM ID: 101608140

Spehar-Délèze, A.-M., Julich, S., Gransee, R., Tomaso, H., Dulay, S.B., O'Sullivan, C.K., 2016. Electrochemiluminescence (ECL) immunosensor for detection of *Francisella tularensis* on screen-printed gold electrode array. Anal. Bioanal. Chem. 408, 7147–7153. doi:10.1007/s00216-016-9658-x

Spibey, N., McCabe, V.J., Greenwood, N.M., Jack, S.C., Sutton, D., van der Waart, L., 2012. Novel bivalent vectored vaccine for control of myxomatosis and rabbit haemorrhagic disease. Vet. Rec. 170, 309. doi:10.1136/vr.100366

Sréter-Lancz, Z., Széll, Z., Sréter, T., Márialigeti, K., 2009. Detection of a novel *Francisella* in *Dermacentor reticulatus*: a need for careful evaluation of PCR-based identification of *Francisella tularensis* in Eurasian ticks. Vector Borne Zoonotic Dis. 9, 123–6. doi:10.1089/vbz.2008.0010

Staples, J.E., Kubota, K.A., Chalcraft, L.G., Mead, P.S., Petersen, J.M., 2006. Epidemiologic and molecular analysis of human tularemia, United States, 1964-2004. Emerg. Infect. Dis. 12, 1113–8. doi:10.3201/eid1207.051504

Steel, M., Penny, D., 2000. Parsimony, likelihood, and the role of models in molecular phylogenetics. Mol. Biol. Evol. 17, 839–850. doi:10.1093/oxfordjournals.molbev.a026364

Steele, S., Radlinski, L., Taft-Benz, S., Brunton, J., Kawula, T.H., 2016. Trogocytosis-associated cell to cell spread of intracellular bacterial pathogens. Elife 5. doi:10.7554/eLife.10625

Stoercklé-Berger N., Keller-Berger B., Ackermann M., Ehrensperger F., 1992. Immunohistological diagnosis of rabbit haemorrhagic disease (RHD). J Vet. Med. [B], 39, 237–245

Strimmer, K., von Haeseler, A., 2009. Genetic distances and nucleotide substitution models. phylogenetic Handb. A Pract. approach to phylogenetic Anal. hypothesis Test. 111–125

Strimmer, K.S., 1997. Maximum Likelihood Methods in Molecular Phylogenetics. Fak. für Biol. 56

Strive, T., Wright, J., Kovaliski, J., Botti, G., Capucci, L., 2010. The non-pathogenic Australian lagovirus RCV-A1 causes a prolonged infection and elicits partial cross-protection to rabbit haemorrhagic disease virus. Virology 398, 125–34. doi:10.1016/j.virol.2009.11.045

Strive, T., Wright, J.D., Robinson, A.J., 2009. Identification and partial characterisation of a new lagovirus in Australian wild rabbits. Virology 384, 97–105. doi:10.1016/j.virol.2008.11.004

Stewart, E. J., 2012. Growing unculturable bacteria. J. Bacteriol. 194, 4151–4160. doi: 10.1128/JB.00345-12

Suitor, E.C., Weiss, E., 1961. Isolation af a *Rickettsia*-like microorganism (Wolbachia Persica, N. SP.) From Argas Persicus (Oken). J. Infect. Dis. 108, 95–106. doi:10.1093/infdis/108.1.95

Suzuki, J., Hashino, M., Matsumoto, S., Takano, A., Kawabata, H., Takada, N., Andoh, M., Oikawa, Y., Kajita, H., Uda, A., Watanabe, K., Shimizu, T., Watarai, M., 2016. Detection of *Francisella tularensis* and analysis of bacterial growth in ticks in Japan. Lett. Appl. Microbiol. 63, 240–6. doi:10.1111/lam.12616

Svensson, K., Bäck, E., Eliasson, H., Berglund, L., Granberg, M., Karlsson, L., Larsson, P., Forsman, M., Johansson, A., 2009. Landscape epidemiology of tularemia outbreaks in Sweden. Emerg. Infect. Dis. 15, 1937–47. doi:10.3201/eid1512.090487

Swofford D.L., Olsen G.J., Waddell P.J., Hillis D.M., 1996. Chapter 11: Phylogenetic inference. Molecular Systematics. 2. Sinauer Associates, Inc., Sunderland. pp426-46

Tamura, K., Nei, M., Kumar, S., 2004. Prospects for inferring very large phylogenies by using the neighbor-joining method. Proc. Natl. Acad. Sci. 101, 11030–11035. doi:10.1073/pnas.0404206101

Tamura, K., Nei, M., 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Mol. Biol. Evol. 10, 512–26. doi:10.1093/molbev/msl149

Tärnvik, A., 2007. WHO Guidelines on Tularaemia. Geneva: WHO/CDS/EPR/2007.7

Tärnvik, A., Sandström, G., Sjöstedt, A., 1996. Epidemiological analysis of tularemia in Sweden 1931-1993. FEMS Immunol. Med. Microbiol. 13, 201–4

Taube, S., Jiang, M., Wobus, C.E., 2010. Glycosphingolipids as receptors for non-enveloped viruses. Viruses 2, 1011–1049. doi:10.3390/v2041011

Tavaré, S., 1986. Some probabilistic and statistical problems in the anal- ysis of DNA sequences. In: Miura R. M., (Ed). Lectures on math- ematics in the life sciences. Volume 17. Providence (RI):American Mathematical Society. pp. 57–86

Thackray, L.B., Wobus, C.E., Chachu, K.A., Liu, B., Alegre, E.R., Henderson, K.S., Kelley, S.T., Virgin, H.W., 2007. Murine noroviruses comprising a single genogroup exhibit biological diversity despite limited sequence divergence. J. Virol. 81, 10460–10473. doi:10.1128/JVI.00783-07

Tham, K.M., Barnes, S.M. and Hunter, S.N., 1999. Polymerase chain reaction amplification and gene sequence analysis of a calicivirus from a feral rabbit. Virus Genes. 1999;18(3):235-42

Thelaus, J., Andersson, A., Broman, T., Bäckman, S., Granberg, M., Karlsson, L., Kuoppa, K., Larsson, E., Lundmark, E., Lundström, J.O., Mathisen, P., Näslund, J., Schäfer, M., Wahab, T., Forsman, M., 2014. *Francisella tularensis* subspecies *holarctica* occurs in Swedish mosquitoes, persists through the developmental stages of laboratory-infected mosquitoes and is transmissible during blood feeding. Microb. Ecol. 67, 96–107. doi:10.1007/s00248-013-0285-1

Thouvenin, E., Laurent, S., Madelaine, M.F., Rasschaert, D., Vautherot, J.F., Hewat, E.A., 1997. Bivalent binding of a neutralising antibody to a calicivirus involves the torsional flexibility of the antibody hinge. J. Mol. Biol. 270, 238–46. doi:10.1006/jmbi.1997.1095

Thumfart, J.O., Meyers, G., 2002. Rabbit hemorrhagic disease virus: identification of a cleavage site in the viral polyprotein that is not processed by the known calicivirus protease. Virology 304, 352–363. doi:10.1006/viro.2002.1660

Tian, L., Liao, J., Li, J., Zhou, W., Zhang, X., Wang, H., 2007. Isolation and identification of a non-haemagglutinating strain of rabbit hemorrhagic disease virus from China and sequence analysis for the VP60 Gene. Virus Genes 35, 745–752. doi:10.1007/s11262-007-0155-3

Tomanović, S., Chochlakis, D., Radulović, Z., Milutinović, M., Cakić, S., Mihaljica, D., Tselentis, Y., Psaroulaki, A., 2013. Analysis of pathogen co-occurrence in host-seeking adult hard ticks from Serbia. Exp. Appl. Acarol. 59, 367–76. doi:10.1007/s10493-012-9597-y

Torres, J.M., Ramírez, M.A., Morales, M., Bárcena, J., Vázquez, B., Espuña, E., Pagès-Manté, A., Sánchez-Vizcaíno, J.M., 2000. Safety evaluation of a recombinant myxoma-RHDV virus inducing horizontal transmissible protection against myxomatosis and rabbit haemorrhagic disease. Vaccine 19, 174–82

Trout, R.C., Tittensor, A.M., 1989. Can predators regulate wild rabbit *Oryctolagus cuniculus* population density in England and Wales? Mamm. Rev. 19, 153–173. doi:10.1111/j.1365-2907.1989.tb00409.x

Valícek, L., Smíd, B., Rodák, L., Kudrna, J., 1990. Electron and immunoelectron microscopy of rabbit haemorrhagic disease virus (RHDV). Arch. Virol. 112, 271–5

Vallat, B., Thiermann, A., Jebara, K. Ben, Dehove, A., 2013. Notification of animal and human diseases : the global legal basis OIE notification system. Rev. Sci. Tech. 32, 331–335

van de Bildt, M.W.G., van Bolhuis, G.H., van Zijderveld, F., van Riel, D., Drees, J.M., Osterhaus, A.D.M.E., Kuiken, T., 2006. Confirmation and phylogenetic analysis of rabbit hemorrhagic disease virus in free-living rabbits from the Netherlands. J. Wildl. Dis. 42, 808–812. doi:10.7589/0090-3558-42.4.808

van der Loo, W., Mougel, F., Sánchez, M.S., Bouton, C., Castien, E., Fonseca, A., Ferrand, N., Soriguer, R., Monnerot, M., 1999. Cytonuclear disequilibria in wild populations of rabbit (*Oryctolagus cuniculus* L.) suggest unequal allele turnover rates at the b locus (IGKC1). Immunogenetics 49, 629–43

van der Loo, W., Ferrand, N., Soriguer, R.C., 1991. Estimation of gene diversity at the b locus of the constant region of the immunoglobulin light chain in natural populations of European rabbit (*Oryctolagus cuniculus*) in Portugal, Andalusia and on the Azorean Islands. Genetics 127, 789–99

van der Loo W., 1987. Studies on the adaptive significance of the immunoglobulin alleles (Ig allotypes) in wild rabbit. In: The Rabbit in Contemporary Immunological Research (ed. Dubiski S),pp. 164-190. Longman Scientific & Technical, John Wiley & Sons, New York

van der Loo W., Arthur C.P., Drees-Wallage M., Richardson B., 1987. Nonrandom allele associations between unlinked protein loci: are the polymorphisms of the immunoglobulin constant regions adaptive? Proceedings of the National Academy of Sciences, USA 84, 3075-3079

Vartoukian, S. R., Palmer, R. M., and Wade, W. G., 2010. Strategies for the culture of "unculturable" bacteria. FEMS Microbiol. Lett. 309, 1–7. doi: 10.1111/j.1574-6968.2010.02000.x

Vashist, S., Bailey, D., Putics, A., Goodfellow, I., 2009. Model systems for the study of human norovirus biology. Future Virol. 4, 353–367. doi:10.2217/fvl.09.18

Versage, J.L., Severin, D.D.M., Chu, M.C., Petersen, J.M., 2003. Development of a multitarget real-time TaqMan PCR assay for enhanced detection of *Francisella tularensis* in complex specimens. J. Clin. Microbiol. 41, 5492–9

Vigne J.D., Alcover J.A., 1985. Incidence des relations historiques entre l'homme et l'animal dans la composition actuelle du peuplement amphibien, reptilien et mammalien des îles de Méditerranée occidentale. I /Oè Congrès National des Sociétés Savantes, section des Sciences, Montpellier, Fasc II, Biologie animale et biologie végétale: 79-91

Vigne J.D., 1988. Données préliminaires sur l'histoire du peuplement mammalian de l'ilot de Zembra (Tunisie). Mammalia 52, 567-574

Villafuerte R., Delibes-Mateos M., 2007. El conejo. In: Palomo LJ, Gisbert J, Blanco JC (eds) Atlas y Libro Rojo de los Mamíferos Terrestres de España. Ministerio de Medioambiente-Dirección General para la Biodiversidad-SECEM, Madrid, pp 490–491

Villafuerte R., Lazo A., Moreno S., 1997. Influence of food abundance and quality on rabbit fluctuations: conservation and management implications in donana national park (sw spain). Rev. Ecol. (Terre Vie), vol. 52

Villafuerte R., Calvete C., Blanco J.C., Lucientes J., 1995. Incidence of viral hemorrhagic disease in wild rabbit populations in Spain. Mammalia, 59:651-660.

Virgós, E., Cabezas-Díaz, S., Lozano, J., 2007. Is the wild rabbit (*Oryctolagus cuniculus*) a threatened species in spain? Sociological constraints in the conservation of specieBiodivers Conserv. 16: 3489-

3504. Chapman & Hall. https://doi.org/10.1007/s10531-006-9054-5

Virgós, E., Cabezas-Díaz, S., Malo, A., Lozano, J., López-Huertas, D., 2003. Factors shaping European rabbit abundance. Acta Theriol. (Warsz). 48, 113–122. doi:10.1007/BF03194271

Vogler, A.J., Birdsell, D., Price, L.B., Bowers, J.R., Beckstrom-Sternberg, S.M., Auerbach, R.K., Beckstrom-Sternberg, J.S., Johansson, A., Clare, A., Buchhagen, J.L., Petersen, J.M., Pearson, T., Vaissaire, J., Dempsey, M.P., Foxall, P., Engelthaler, D.M., Wagner, D.M., Keim, P., 2009. Phylogeography of Francisella tularensis: Global Expansion of a Highly Fit Clone. J. Bacteriol. 191, 2474–2484. doi:10.1128/JB.01786-08

Wallet, P., Lagrange, B., Henry, T., 2016. *Francisella* inflammasomes: Integrated responses to a cytosolic stealth bacterium. Curr. Top. Microbiol. Immunol. 397, 229–256. doi:10.1007/978-3-319-41171-2_12

Wang, Y., Peng, Y., Hai, R., Xia, L., Li, H., Zhang, Z., Cai, H., Liang, Y., Shen, X., Yu, D., Birdsell, D., Wagner, D.M., Keim, P., 2014. Diversity of *Francisella tularensis* subsp. *holarctica* lineages, China. Emerg. Infect. Dis. 20, 1191–4. doi:10.3201/eid2007.130931

Wang, X., Xu, F., Liu, J., Gao, B., Liu, Y., Zhai, Y., Ma, J., Zhang, K., Baker, T.S., Schulten, K., Zheng, D., Pang, H., Sun, F., 2013. Atomic model of rabbit hemorrhagic disease virus by cryo-electron microscopy and crystallography. PLoS Pathog. 9, e1003132. doi:10.1371/journal.ppat.1003132

Ward D., 2005. Reversing rabbit decline: one of the biggest challenges for nature conservation in Spain and Portugal. IUCN Technical Report. Available at: http://www.ualberta.ca/~dhik/lsg/report_lynx_rabit.pdf

Wehrly, T.D., Chong, A., Virtaneva, K., Sturdevant, D.E., Child, R., Edwards, J.A., Brouwer, D., Nair, V., Fischer, E.R., Wicke, L., Curda, A.J., Kupko, J.J., Martens, C., Crane, D.D., Bosio, C.M., Porcella, S.F., Celli, J., 2009. Intracellular biology and virulence determinants of *Francisella tularensis* revealed by transcriptional profiling inside macrophages. Cell. Microbiol. 11, 1128–1150. doi:10.1111/j.1462-5822.2009.01316.x

Weinert, L.A., Werren, J.H., Aebi, A., Stone, G.N., Jiggins, F.M., 2009. Evolution and diversity of *Rickettsia* bacteria. BMC Biol. 7, 6. doi:10.1186/1741-7007-7-6

Weller, S.J., Baldridge, G.D., Munderloh, U.G., Noda, H., Simser, J., Kurtti, T.J., 1998. Phylogenetic placement of rickettsiae from the ticks *Amblyomma americanum* and *Ixodes scapularis*. J. Clin. Microbiol. 36, 1305–17

Westcott, D.G., Frossard, J.-P., Everest, D., Dastjerdi, A., Duff, J.P., Choudhury, B., 2014. Incursion of RHDV2-like variant in Great Britain. Vet. Rec. 174, 333–333. doi:10.1136/vr.g2345

White, P.J., Norman, R.A., Hudson, P.J., 2002. Epidemiological consequences of a pathogen having both virulent and avirulent modes of transmission: the case of rabbit haemorrhagic disease virus. Epidemiol. Infect. 129, 665–77

White, P.J., Norman, R.A., Trout, R.C., Gould, E.A., Hudson, P.J., 2001. The emergence of rabbit haemorrhagic disease virus: will a non-pathogenic strain protect the UK? Philos. Trans. R. Soc. B Biol. Sci. 356, 1087–1095. doi:10.1098/rstb.2001.0897

Williams, D., Acevedo, P., Gortazar, C., Escudero, M.A., Labarta, J.L., Marco, J., Villafuerte, R., 2007.

Hunting for answers: Rabbit (*Oryctolagus cuniculus*) population trends in northeastern Spain. Eur. J. Wildl. Res. 53, 19–28. doi:10.1007/s10344-006-0056-0

Wilson, M.E., 1995. Travel and the emergence of infectious diseases. Emerg. Infect. Dis. 1, 39–46. doi:10.3201/eid0102.952001

Wirblich, C., Meyers, G., Ohlinger, V.F., Capucci, L., Eskens, U., Haas, B., Thiel, H.J., 1994. European brown hare syndrome virus: relationship to rabbit hemorrhagic disease virus and other caliciviruses. J. Virol. 68, 5164–73

Wirblich, C., Thiel, H.J., Meyers, G., 1996. Genetic map of the calicivirus rabbit hemorrhagic disease virus as deduced from in vitro translation studies. J. Virol. 70, 7974–83. doi:10.1006/viro.2000.0579

Woolhouse, M.E.J., Webster, J.P., Domingo, E., Charlesworth, B., Levin, B.R., 2002. Biological and biomedical implications of the co-evolution of pathogens and their hosts. Nat. Genet. 32, 569–577. doi:10.1038/ng1202-569

Wu, X., Ren, G., Gunning, W.T., Weaver, D.A., Kalinoski, A.L., Khuder, S.A., Huntley, J.F., 2016. FmvB: A *Francisella tularensis* magnesium-responsive outer membrane protein that plays a role in virulence. PLoS One 11, e0160977. doi:10.1371/journal.pone.0160977

Xu, W.Y., 1991. Viral haemorrhagic disease of rabbits in the People's Republic of China: epidemiology and virus characterisation. Rev. Sci. Tech. 10, 393–408

Xu, Z.J., Chen, W.X., 1989. Viral haemomhagic disease in rabbits: a review. Vet. Res. Commun., 13, 205-212

Yang, L., Wang, F., Hu, B., Xue, J., Hu, Y., Zhou, B., Wang, D. and Xu, W., 2008. Development of an RT-PCR for rabbit hemorrhagic disease virus (RHDV) and the epidemiology of RHDV in three eastern provinces of China. J Virol Methods. 151, 24-9.

Yang, Z., 2007. PAML 4: Phylogenetic analysis by maximum likelihood. Mol. Biol. Evol. 24, 1586–1591. doi:10.1093/molbev/msm088

Yang, H.C., 1989a. A study on detecting RHD antibodies by the indirect haemagglutination test (in Chinese). Anim. Hus. vet. Med., 21(3) 100-10

Yang, H.C., Xu Y., Du N. X., 1989b. Studies on the haemagglutination characteristics of virus of rabbit haemorrhagic disease (*in Chinese*). Anim. Hus. vet. Med., 21(1): 23-26

Yang, Z., Rannala, B., 1997. Bayesian phylogenetic inference using DNA sequences: a Markov Chain Monte Carlo Method. Mol. Biol. Evol. 14, 717–24

Yesilyurt, M., Kilic, S., Celebi, B., Celik, M., Gul, S., Erdogan, F., Ozel, G., 2011. Antimicrobial susceptibilities of *Francisella tularensis* subsp. *holarctica* strains isolated from humans in the Central Anatolia region of Turkey. J. Antimicrob. Chemother. 66, 2588–2592. doi:10.1093/jac/dkr338

Yuan, D., Guo, D., Liu, J., Si, C., Jiang, Q., Lin, H., Yang, T., Qu, L., 2013. Development of a reverse-transcription loop-mediated isothermal amplification method for detection of rabbit hemorrhagic disease virus. J. Virol. Methods 187, 274–7. doi:10.1016/j.jviromet.2012.11.020

Ziveri, J., Barel, M., Charbit, A., 2017. Importance of Metabolic Adaptations in *Francisella* Pathogenesis. Front. Cell. Infect. Microbiol. 7, 96. doi:10.3389/fcimb.2017.00096

6. Annexes

Annex I

Tularémia

TULARÉMIA

Isabel Lopes de Carvalho, Carina Carvalho, Maria Sofia Núncio

Introdução

A tularémia é uma zoonose causada pela bactéria *Francisella tularensis*. O agente etiológico é transmitido ao Homem por contacto direto com animais infetados, ar, água, alimentos contaminados ou por vetores hematófagos [1,2,3]. A primeira descrição desta bactéria ocorreu em 1912 no condado de Tulare, Califórnia, por George McCoy e Charles Chapin. Inicialmente, esta bactéria foi denominada *Bacterium tularense* sendo posteriormente designada *F. tularensis* em honra a Edward Francis, investigador que isolou o organismo pela primeira vez. Desde então tem sido descrita em várias localizações no Hemisfério Norte [1,2,3,4].

Com a emergência de *F. tularensis* em novos locais e populações e com o seu potencial uso em bioterrorismo, uma vez que se encontra classificado na lista de agentes de classe A do *Centers for Disease Control and Prevention* (CDC), no último século o interesse da comunidade cientifica nesta bactéria cresceu e por isso o seu estudo tem-se intensificado [4,5].

Em Portugal, só em 1998, na sequência de um surto epidémico em Espanha, a Direcção Geral de Saúde emitiu um comunicado alertando os clínicos para a possibilidade da ocorrência desta zoonose no nosso país. Desde então o diagnóstico laboratorial desta patologia foi implementado e atualizado no CEVDI/INSA [1].

Taxonomia e distribuição

F. tularensis é um pequeno cocobacilo Gram-negativo, pleomórfico, aeróbico, catalase-positivo, não móvel [2].

Francisella spp. pertence ao grupo gamma (γ)-proteobacteria e é classificada com base em características de crescimento, reações bioquímicas e propriedades de virulência [2,3]. A família *Francisellaceae* inclui três espécies do género *Francisella*, *F. hispaniensis*, *F. philomiragia* e *F. tularensis*. A espécie *F. tularensis* engloba três subespécies com diferentes níveis de patogenicidade e distribuição geográfica: *F tularensis* subsp. *holarctica*, *F. tularensis* subsp. *mediasiatica* e *F. tularensis* subsp.

tularensis [2,6]. Atualmente, a espécie *F. novicida* é considerada a quarta subespécie de *F. tularensis* [3,6,7,8,9,10]. No entanto, algumas objeções têm surgido a esta classificação com base em resultados de sequenciação do genoma que mostram uma evolução divergente das duas populações [11].

A tularémia é uma doença amplamente distribuída no Hemisfério Norte, com focos em algumas partes da América do Norte, Europa e Norte da Ásia. *F. tularensis* subsp. *tularensis* é encontrada predominantemente na América do Norte, apesar de já ter sido isolada na Europa [1,4]. *F. tularensis* subsp. *holarctica* está distribuida por todo o Hemisfério Norte e, recentemente, foi também detectada na Tâsmania (Austrália) [1,3,12]. *F tularensis* subsp. *mediasiatica* apresenta uma distribuição restrita à Ásia Central e *F. novicida* foi isolada na América do Norte, na Austrália e na Tailândia [3,7,13].

Na natureza, a infeção por *F. tularensis* é encontrada numa variedade de espécies animais incluindo lagomorfos, roedores, insectívoros, carnívoros, ungulados, marsupiais, aves, anfíbios, peixes e invertebrados. Os artrópodes, incluindo ixodídeos (*Dermacentor* spp., *Ixodes* spp. e *Amblyomma americanum*) e mosquitos (*Aedes*, *Culex* e *Anopheles*) são potenciais vetores. Os roedores e lagomorfos são apontados como os principais reservatórios desta bactéria [8,14,15,16].

As principais vias de transmissão incluem o contacto direto com tecidos ou fluidos de animais infetados, a picada de artrópodes e a ingestão de água ou comida contaminada. A transmissão pessoa a pessoa nunca foi descrita [2,17].

A verdadeira incidência de tularémia é desconhecida porque muitas vezes os casos não são notificados. Na Europa, em 2003 foi emitido um comunicado para que esta doença passasse a ser uma doença com vigilância epidemiológica (Decisão n° 2000/96/EC). Em Portugal, até à data não há nenhum caso notificado, no entanto desde 1997 que o CEVDI/INSA tem o diagnóstico laboratorial estabelecido, recebendo em média por ano cerca de 20 novos pedidos.

Patogénese

F. tularensis é uma bactéria intracelular facultativa que pode invadir e multiplicar-se em diferentes tipos de células [3,18,19]. As células apresentadoras de antigénio, como os macrófagos e as células dendríticas são os alvos principais de *F. tularensis* no início da infeção [20]. A virulência da bactéria está diretamente relacionada com a sua capacidade para se replicar no citosol das células infetadas [21].

F. tularensis possui diferentes mecanismos através dos quais subverte a deteção pelo sistema imunitário do hospedeiro, na porta de entrada [21]: a membrana externa apresenta estruturas modificadas que lhe permitem evitar a interação com os recetores do hospedeiro associado à indução de inflamação; utiliza células alvo que não dispõem dos co-recetores que facilitam a ligação aos recetores que alertam as células do hospedeiro para a infeção; utiliza recetores que falham na iniciação da produção de citocinas pro-inflamatórias.

Diagnóstico clínico

F. tularensis pode infetar o homem através da pele, inalação, mucosas e via gastrointestinal. O estabelecimento desta infeção depende da porta de entrada e do número de organismos [2,3,17]. O resultado pode variar desde casos assintomáticos a uma septicémia aguda seguida de morte rápida. As principais apresentações de doença incluem as formas ulceroglandular, glandular, oculoglandular, orofaringea, pneumónica e tífica [2].

O período de incubação é habitualmente de 3 a 5 dias, mas pode variar entre um e 21 dias. O início de doença é usualmente brusco, com febre (38-40°C), cefaleias, arrepios de frio, rigidez da nuca, mialgias (predominantemente lombares), síndroma tipo gripal e odinofagia. Tosse seca e dor ou aperto retrosternais podem ocorrer associados a sinais objetivos de pneumonia, tais como expetoração, dispneia, taquipneia, dor na pleura ou hemoptise. Algumas vezes ocorrem ainda náuseas, vómitos e diarreia. A fase subsequente é caracterizada por suores, febre e arrepios de frio, astenia, anorexia e perda de peso [2].

Na forma ulceroglandular, a manifestação típica que surge depois do manuseamento de carcaças contaminadas ou após a picada de um artrópode infetado consiste numa pápula cutânea no local de inoculação, em simultâneo com outros sintomas generalizados, e que se torna purulenta e ulcerada poucos dias após o seu aparecimento. A úlcera é mole, geralmente tem um carácter indolor e pode vir a cobrir-se por uma escara. Tipicamente, um ou mais nódulos linfáticos aferentes podem tornar-se maiores e moles poucos dias após o aparecimento da pápula [1,2].

Na forma oculoglandular, após a contaminação direta do olho ocorre ulceração da conjuntiva, acompanhada de equimoses, vasculite e linfadenite localizadas [1,2].

A forma glandular de tularémia é caracterizada por deteção de linfadenopatia sem úlcera [1,2].

A forma orofaríngea é adquirida pela ingestão de água ou alimentos contaminados e, algumas vezes, pela inalação de aerossóis. Algumas das pessoas infetadas podem desenvolver estomatites, mas geralmente desenvolvem faringite exsudativa ou amigdalite, algumas vezes com ulceração. Pode ainda ocorrer linfadenopatia cervical ou retrofaríngea pronunciada [1,2].

A tularémia pneumónica pode ser o resultado direto da inalação de aerossóis contaminados ou seguir-se à disseminação hematogénica a partir de um outro local do organismo. A libertação destes aerossóis pode resultar em doença aguda, com sinais e sintomas de faringite, bronquite, pleuropneumonia e linfadenite, acompanhada por manifestações de doença sistémica. No entanto, as exposições por inalação resultam usualmente num quadro clínico inicial de doença sistémica sem sinais claros de doença respiratória [1,2].

A designação de forma tífica aplica-se para descrever uma doença sistémica, cujo diagnóstico se pode confundir com a febre tifóide, com ausência de sinais no local de infeção. Algumas vezes, estes doentes apresentam manifestações gastrointestinais intensas, tais como diarreia e cólicas [1,2].

Diagnóstico laboratorial

O tipo de amostra a utilizar no diagnóstico laboratorial depende das manifestações clínicas que o doente apresenta e pode incluir esfregaços da lesão ou zaragatoas, sangue total, urina, biópsia, aspirado ou raspagem (úlcera, nódulo linfático, córnea ou tecido afetado) e amostras respiratórias (expetoração, lavado bronquial ou pleural) [1,17]. Num contexto de surto epidémico ou em estudos epidemiológicos, devem ser estudados artrópodes potenciais vetores, carcaças dos animais, fezes dos hospedeiros vertebrados e amostras de água [17].

De acordo com o CDC, a cultura continua a ser o *gold standard* para a confirmação laboratorial de infeção por *F. tularensis* [1,2,17]. Contudo, tanto *F. tularensis* subsp. tularensis e *F. tularensis* subsp. *holarctica* têm um crescimento lento e são organismos exigentes que requerem 24-72h e temperatura de 37°C para crescer em meio artificial [22].

F. tularensis pode ser cultivada in vitro em meio de cultura líquido ou sólido adequado [22]. Habitualmente, as estirpes de *F. tularensis* isoladas a partir de amostras clínicas desenvolvem-se bem em diversos meios incluindo agar de chocolate (CA), agar de cisteína enriquecido com sangue achocolatado (9%) (CHAB) e agar de extrato

de fermento de carvão tamponado (BYCE) [24]. O meio CHAB é o mais recomendado por permitir a identificação presuntiva de *F. tularensis*: este microrganismo apresenta um crescimento característico neste meio (verde opalescente com colónias brilhantes) às 24-48 horas [17,22,23]. Uma vez em presença do isolado puro, a fermentação por glicerol pode ser usada para diferenciar tipo A (*F. tularensis* subsp. *tularensis*) - faz a fermentação de glicerol - de tipo B (*F. tularensis* subsp. *holarctica*) - não faz fermentação de glicerol [23].

Esta bactéria é conhecida por causar infeções laboratoriais, pelo que o manuseamento de culturas e tentativa de isolamento só podem ser realizados em condições de biossegurança de nível 3 (BSL-3). Apesar desta limitação, o isolamento do agente continua a ser o método mais recomendado, pois permite o diagnóstico definitivo e constitui um valioso recurso para a epidemiologia molecular [17].

Os estudos serológicos são a forma mais comum de confirmar o diagnóstico de tularémia. Esta confirmação requer a observação de seroconversão, ou seja, a deteção de um aumento de quatro vezes, ou mais, dos títulos de anticorpos específicos presentes em duas amostras de soro consecutivas colhidas, respetivamente, na fase aguda e convalescente da doença [2,23,24].

Ao nível do diagnóstico indireto, estão disponíveis as técnicas de aglutinação em tubo, microaglutinação e *Enzyme-linked immunosorbent assays* (ELISA) para deteção de anticorpos contra *F. tularensis* [25,26].

A técnica de microaglutinação é 100 vezes mais sensível do que o método de aglutinação em tubo. Os anticorpos IgM e IgG são detetados em simultâneo e é habitual persistirem com títulos elevados por mais de uma década após a infeção, limitando o valor de um só resultado positivo. A técnica de ELISA é referida como sendo mais sensível do que os métodos de aglutinação e tem a vantagem de detetar separadamente diferentes classes de anticorpos [2,23].

O uso da técnica de *Polymerase chain reaction* (PCR) é muito útil quando as culturas são negativas ou o isolamento microbiológico é impraticável por não estarem reunidas as condições de biossegurança exigidas para a execução desta técnica [2,17].

Os genes que codificam a liproproteína da membrana externa (*tul4*) de *F. tularensis* foram o primeiro alvo no desenvolvimento da técnica de PCR aplicada à deteção do agente em amostras de sangue, aerossóis e tecidos de animais. Este protocolo, seguido pela confirmação por sequenciação, RLB (*reverse line blotting*) ou por RFLP (*restriction fragment length polymorphism*), mostrou ser mais sensível que a cultura, embora seja menos sensível que a técnica de PCR em tempo real (RT-PCR) [23].

Neste momento o protocolo de RT-PCR Taq-Man ™ usado no diagnóstico laboratorial e em investigação tem como alvo três genes (*ISFtu2, tul4, fopA*) que para além de aumentar a sensibilidade para um limite de deteção de ~1 CFU, tem a vantagem de diminuir a probabilidade de aparecimento de falsos negativos [23, 27].

Uma vez que a diferenciação entre *F. tularensis* subsp. *tularensis* e *F. tularensis* subsp. *holarctica* apresenta um grande interesse clínico foi desenvolvido um protocolo de PCR em tempo real que possibilita esta discriminação; este protocolo usa sondas TaqMan™ e é direcionado a uma região divergente de uma "ilha de patogenia" denominada *Francisella patogenicity island* (FIP) [27,28]. A genotipagem feita pela análise de MVLA (*multiple-locus variable-number tandem repeat analysis*) permite ter mais detalhe nos aspetos ecológicos e é a base para os estudos de diferenciação molecular das subespécies de *F. tularensis* [29].

Situação em Portugal

Em Portugal, a ocorrência de casos humanos de tularémia nunca foi oficialmente notificada, pelo que a doença apresenta, muito provavelmente, uma prevalência subestimada. Isto deve-se, em parte, ao facto desta doença ser ainda pouco divulgada em Portugal e ao facto de existir pouca informação disponível para a população de risco e para os técnicos de saúde [1]. Contudo, já foram realizados estudos seroepidemiológicos onde foi possível detetar a presença de anticorpos anti-*F. tularensis* na população portuguesa [30, 31].

Anualmente é solicitado ao INSA o diagnóstico laboratorial desta doença em cerca de duas dezenas de casos. Em 1998, na sequência de um surto epidémico em Espanha, a Direção Geral de Saúde emitiu um comunicado alertando os clínicos para a possibilidade da tularémia se alastrar ao território nacional, quer por extensão do surto quer por ocorrência de casos isolados, dado o fluxo de caçadores, populações de vetores e animais vertebrados, que não reconhecem as fronteiras desenhadas pelo Homem (Decisão nº 2003/534/CE). A inclusão de *F. tularensis* na lista dos agentes potencialmente utilizáveis em bioterrorismo, juntamente com o caráter endémico que a doença tem em Espanha, relançou o interesse por esta patologia no nosso país [5].

Em 2007, foi detetada pela primeira vez por métodos moleculares, *F. tularensis* subsp. *holarctica* numa amostra humana e num *D. reticulatus* colhido em Bragança, região que faz fronteira com uma das zonas onde já ocorreram surtos em Espanha [5].

Em Portugal, o papel das carraças e dos roedores e leporídeos silvestres, diretamente implicados na transmissão da doença, necessita ainda de ser clarificado. No entanto, os últimos resultados obtidos da investigação em curso indicam que para além de *D. reticulatus*, outras espécies de ixodídeos estão implicados na transmissão de *F. tularensis* e que as carraças são o vetor mais importante da tularémia em Portugal, à semelhança do que acontece noutros países em que a tularémia é endémica. No que se refere aos pequenos mamíferos, *F. tularensis* subsp. *holarctica* foi recentemente detetada em amostras de leporídeos silvestres pela primeira vez em Portugal [32].

Bibliografia

1. Lopes de Carvalho I, Núncio MS, David de Morais J. Tularémia. Acta Med Port. 2009 (3): 281-90.
2. Mandell GL, Bennet JE, Dolin R. Mandel, Douglas and Bennet's Priciples and Practice of Infectious Diseases. Elsevier, Churchil Linvingstone. 2005; (2):2674-2683.
3. Oyston PC. *Francisella tularensis*: unraveling the secrets of an intracellular pathogen. J Med Microbiol. 2008; 57(Pt8):921-39.
4. Petersen JM, Schriefer ME. Tularemia: emergence/re-emergence. Vet Res. 2005; 36(3):455-67.
5. Lopes de Carvalho I, Escudero R, Garcia-Amil C, Falcão H, Anda P, Núncio MS. *Francisella tularensis*, Portugal. Emerg Infect Dis. 2007; 13(4):666-7.
6. Hansen CM, Vogler AJ, Keim P, Wagner DM, Hueffer K. Tularemia in Alaska, 1938-2010. Acta Vet Scand. 2011; 18(53):61.
7. Vogler AJ, Birdsell D, Price LB, Bowers JR, Beckstrom-Sternberg SM, Auerbach RK, Beckstrom-Sternberg JS, Johansson, Clare A, Buchhagen JL, Petersen JM, Pearson, Vaissaire J, Dempsey MP, Foxall P, Engelthaler DM, Wagner DM, Keim P. Phylogeography os *Francisella tularensis*: Global Expansion of a Highly Fit Clone. J Bacteriol. 2009; 191(8):2474-84
8. Ellis J, Oyston PCF, Green M, Titball RW. Tularemia. Clin Microbiol Rev. 2002; (15):629-46.
9. Huber B, Escudero R, Busse HJ, Seibold E, Scholz HC, Anda P, Kämpfer P, Splettsoesser WD. Description of *Francisella hispaniensis* sp.nov., isolated from human blood, reclassification of *Francisella novicida* (Larson et al. 1955) Olsufiev et al 1959 as *Francisella tularensis* subspecies *novicida* comb. nov. and emended description of the genus *Francisella*. Int J Syst Evol Microbiol. 2010; 60(Pt8):1887-96.
10. Siddaramappa S, Challacombe JF, Petersen JM, Pillai S, Hogg G, Kuske CR. Commom ancestry and novel genetic traits of *Francisella novicida*-like isolates from North America and Australia as revealed by comparative genomic analysis. Appl Environ Microbiol. 2011; 77(15):5110-22.
11. Johansson A, Celli J, Conland W, Elkins KL, Forsman M, keim PS, Larsson P, Manoil C, Nano FE, Petersen JM, Sjösted A. Objections to the transfer of *Francisella novicida* to the subspecies rank of *Francisella tularensis*. Int J Syst Evol Microbiol. 2010; 60(Pt8):1717-8.
12. Jackson J, McGregor A, Cooley L, Ng J, Brown M, Ong CW, Darci C, Sintchenko V. *Francisella tularensis* subspecies *holarctica*, Tasmania, Australia, 2011. Emerg Infect Dis. 2012; 18(9):1484-6.
13. Whipp MJ, Davis JM, Lum G, de Boer J, Zhou Y, Bearden SW, Petersen JM, Chu MC, Hogg G. Characterization of a *novicida*-like subspecies of *Francisella tularensis* isolated in Australia. J Med Microbiol. 2003; 52(Pt9):893-42.

14. Petersen JM, Mead PS, Schriefer ME. *Francisella tularensis*: an arthropod-borne pathogen. Vet Res. 2009; 40(2):7.

15. Gyuranecz M, Szeredj L, Makrai L, Fodor L, Mészáros AR, Szépe B, Füleki M, Erdélvi K. Tularemia of Brown European Brown Hare (*Lepus europaeus*): a pathological, histopatological and immunohistochemical study. Vet Pathol. 2010; 47(5):958-63.

16. Kuehn A, Shulze C, Kutzer P, Probst C, Hlinak A, Ochs A, Grunow R. Tularaemia seroprevalence of captured and wild animals in Germany: the fox (*Vulpes vulpes*) as a biological indicator. Epidemiol Infect. 2013; 141(4):833-40.

17. World Health Organization working group on Tularaemia. WHO Guidelines on Tularaemia. 2007. pp: 115

18. Foley JE, Nieto NC. Tularemia. Vet Microbiol. 2010; 140(3-4):332-8.

19. Cowley SC, Elkins KL. Immunity to *Francisella*. Front Microbiol. 2011; 2:26.

20. Bosio CM. The subversion of the immune system by *Francisella tularensis*. Front Microbiol. 2011; 2:9.

21. Henry T, Brotcke A, Weiss DS, Thompson LJ, Monack DM. Type I interferon signaling is required for activation of the inflammasome during *Francisella* infection. J Exp Med. 2007; 204(5): 987-94.

22. Murray PR, Baron EJ, Pfaller MA, Tenover FC, Yolken RH. Manual of clinical microbiology, 7th ed. Washington DC: ASM press. 1999.

23. Splettstoesser WD, Tomaso H, Al Dahouk S, Neubaeur H, Schuff-Werner P. Diagnostic procedures in tularemia with special focus on molecular and immunological techniques. J Vet Med B Infect Dis Vet Public Health. 2005; 52(6):249-61.

24. Tärnvik A, Berglund L. Tularemia. Eur Resp J. 2003;21(2):361-73.

25. Feldman KA. Tularemia. JAMA. 2003;222(6):725-730.

26. Bevanger L, Maeland JA, Kvan AI. Comparative analyses of antibodies to Francisella tularensis antigens during the acute phase of tularemia and eight years later. Clin Diagn Lab Immunol. 1994. 1:238-240.

27. Versage JL, Severin DD, Chu MC, Petersen JM. Development of a multitarget real-time TaqMan PCR assay for enhanced detection of *Francisella tularensis* in complex specimens. J Clin Microbiol. 2003; 41(12):5492-9.

28. Nano FE, Zhang Na, Cowley SC, Klose KE, Cheung KK, Roberts MJ, Ludu JS, Letendre GW, Meierovics AI, Stephens G, Elkins KL. A *Francisella tularensis* pathogenicity island required for intramacrophage growth. J Bacteriol. 2004; 186 (19):6430-6.

29. Vogler AJ, Birdsell D, Wagner DM, Keim P. An optimized, multiplexed multi-*locus* variable-number tandem repeat analysis system for genotyping *Francisella tularensis.* Lett Appl Microbiol. 2009; 48(1):140-4.

30. Seabra J, Santos MA, Pereira H, Vicente P, Vasconcelos O, Santos A. Prevalence of *Francisella tularensis* antibodies in the population of North Portugal. In: Abstracts of the Prevention and Control of Zoonoses: Cardiff, Wales, UK; 2002. Cardiff (wales); Health Protection Agency; 2002.

31. Lopes de Carvalho I. Aplicação de técnicas imunológicas e moleculares ao estudo de *Francisella tularensis* em Portugal. Tese de mestrado em Microbiologia Clinica. Faculdade de Medicina da Universidade de Lisboa, 2008.

32. Lopes de Carvalho I, Carvalho CL, Respicio-Kingry LB, Zé-Zé, Petersen JM, Núncio MS. Assessment of *Francisella tularensis* in Portugal. In: abstracts of the International Symposium on *Francisella tularensis* and tularemia. Urgup, Turkey; 2013.

Annex II

Terrestrial Animal Health Code (2016)

Volume II

Recommendations applicable to OIE Listed diseases and other diseases of importance to international trade

Chapter 8.17.: Tularemia

CHAPTER 8.17.

TULAREMIA

Article 8.17.1.

General provisions

For the purposes of the *Terrestrial Code*, the *incubation period* for tularemia (in hares, genus *Lepus*) shall be 15 days.

Standards for diagnostic tests are described in the *Terrestrial Manual*.

Article 8.17.2.

Tularemia free country

A country may be considered free from tularemia when it has been shown that tularemia has not been present for at least the past two years and when bacteriological or serological surveys in previously infected *zones* have given negative results.

Article 8.17.3.

Tularemia infected zone

A *zone* should be considered as infected with tularemia until:

1) at least one year has elapsed after the last *case* has been confirmed;

AND

2) a bacteriological survey on ticks within the infected *zone* has given negative results; or
3) regular serological testing of hares and rabbits from that *zone* have given negative results.

Article 8.17.4.

Trade in commodities

Veterinary Authorities of tularemia free countries may prohibit importation or transit through their territory, from countries considered infected with tularemia, of live hares.

Article 8.17.5.

Recommendations for importation from countries considered infected with tularemia

For live hares

Veterinary Authorities should require the presentation of an *international veterinary certificate* attesting that the *animals*:

1) showed no clinical sign of tularemia on the day of shipment;
2) were not kept in a tularemia infected *zone*;
3) have been treated against ectoparasites; and
4) were kept in a *quarantine station* for the 15 days prior to shipment.

Annex III

Terrestrial Animal Health Code (2016)

Volume II

Recommendations applicable to OIE Listed diseases and other diseases of importance to international trade

Chapter 13.2.: Rabbit haemorrhagic disease

CHAPTER 13.2.

RABBIT HAEMORRHAGIC DISEASE

Article 13.2.1.

General provisions

For the purposes of the *Terrestrial Code*, the *infective period* for rabbit haemorrhagic disease (RHD) shall be 60 days.

Standards for diagnostic tests and vaccines are described in the *Terrestrial Manual*.

Article 13.2.2.

RHD free country

A country may be considered free from RHD when it has been shown that the *disease* has not been present for at least one year, that no *vaccination* has been carried out in the previous 12 months, and that virological or serological surveys in both domestic and *wild* rabbits have confirmed the absence of the *disease*.

This period may be reduced to six months after the last *case* has been eliminated and *disinfection* procedures completed in countries adopting a *stamping-out policy*, and where the serological survey confirmed that the *disease* had not occurred in the *wild* rabbits.

Article 13.2.3.

RHD free establishment

An *establishment* may be considered free from RHD when it has been shown, by serological testing, that the *disease* has not been present for at least one year, and that no *vaccination* has been carried out in the previous 12 months. Such *establishments* should be regularly inspected by the *Veterinary Authority*.

A previously infected *establishment* may be considered free when six months have elapsed after the last *case* has been eliminated, and after:

1) a *stamping-out policy* has been adopted and carcasses have been disposed of by burning;
2) the rabbitry has been thoroughly disinfected and kept empty for at least six weeks;
3) the rabbitry is properly fenced to prevent the straying of *wild* lagomorphs into the rabbitry.

Article 13.2.4.

Trade in commodities

Veterinary Authorities of RHD free countries may prohibit importation or transit through their territory, from countries considered infected with RHD, of live rabbits, semen, *meat* and non-treated pelts.

Article 13.2.5.

Recommendations for importation from RHD free countries

For domestic rabbits destined for breeding

Veterinary Authorities of *importing countries* should require the presentation of an *international veterinary certificate* attesting that the *animals*:

1) showed no clinical sign of RHD on the day of shipment;

2) were kept in a RHD free country since birth or for at least the past 60 days.

Article 13.2.6.

Recommendations for importation from RHD free countries

For day-old rabbits destined for breeding

Veterinary Authorities of *importing countries* should require the presentation of an *international veterinary certificate* attesting that the *animals*:

1) showed no clinical sign of RHD on the day of shipment;
2) were born from female rabbits which had been kept in a country free from RHD for at least the past 60 days.

Article 13.2.7.

Recommendations for importation from countries considered infected with RHD

For domestic rabbits destined for breeding or pharmaceutical or surgical or agricultural or industrial use

Veterinary Authorities of *importing countries* should require the presentation of an *international veterinary certificate* attesting that the *animals*:

1) showed no clinical sign of RHD on the day of shipment;

AND

2) were kept in a RHD free *establishment* where no clinical *case* of RHD was found when inspected by an *Official Veterinarian* immediately prior to shipment;

OR

3) were kept in an *establishment* where no *case* of RHD was reported during the 60 days prior to shipment and no clinical *case* of RHD was found when inspected by an *Official Veterinarian* immediately prior to shipment; and
4) were kept in an *establishment* where no *animal* has been vaccinated against RHD; and
5) were kept in an *establishment* where breeding rabbits (at least 10% of the *animals*) were subjected to the serological test for RHD with negative results during the 60 days prior to shipment; and
6) have not been vaccinated against RHD; or
7) were vaccinated against RHD immediately before shipment (the nature of the vaccine used and the date of *vaccination* shall also be stated in the certificate).

Article 13.2.8.

Recommendations for importation from countries considered infected with RHD

For day-old rabbits destined for breeding

Veterinary Authorities of *importing countries* should require the presentation of an *international veterinary certificate* attesting that the *animals*:

1) were kept in a RHD free *establishment* where no clinical *case* of RHD was found when inspected by an *Official Veterinarian* immediately prior to shipment;

OR

2) were kept in an *establishment* where no *case* of RHD was reported during the 30 days prior to shipment and no clinical *case* of RHD was found when inspected by an *Official Veterinarian* immediately before shipment; and
3) have not been vaccinated against RHD; and
4) were born from female rabbits which were subjected to the serological test for RHD with negative results during the 60 days prior to shipment.

Article 13.2.9.

Recommendations for importation from countries considered infected with RHD

For domestic rabbits destined for immediate slaughter

Veterinary Authorities of *importing countries* should require the presentation of an *international veterinary certificate* attesting that the *animals*:

1) showed no clinical sign of RHD on the day of shipment;
2) were kept in an *establishment* where no *case* of RHD was reported during the 60 days prior to shipment.

Article 13.2.10.

Recommendations for importation from countries considered infected with RHD

For semen

Veterinary Authorities of *importing countries* should require the presentation of an *international veterinary certificate* attesting that the donor animals:

1) showed no clinical sign of RHD on the day of collection of the semen;
2) were subjected to the serological test for RHD with negative results during the 30 days prior to collection.

Article 13.2.11.

Recommendations for importation from countries considered infected with RHD

For domestic rabbit meat

Veterinary Authorities of *importing countries* should require the presentation of an *international veterinary certificate* attesting that the *meat* comes from *animals* which:

1) were kept in an *establishment* where no *case* of RHD was reported during the 60 days prior to transport to the approved *abattoir*;
2) were subjected to ante-mortem inspections for RHD with favourable results;
3) showed no lesions of RHD at post-mortem inspections.

Article 13.2.12.

Recommendations for importation from RHD free countries

For non-treated pelts

Veterinary Authorities of *importing countries* should require the presentation of an *international veterinary certificate* attesting that the pelts come from rabbits which had been kept in a country free from RHD for at least 60 days before *slaughter*.

Article 13.2.13.

Recommendations for importation from countries considered infected with RHD

For pelts

Veterinary Authorities of *importing countries* should require the presentation of an *international veterinary certificate* attesting that the pelts were subjected to a drying treatment for at least one month and a formalin-based treatment by spraying at a 3% concentration, or by fumigation carried out, not more than seven days prior to shipment.

Annex IV

Standard Method Performance Requirements (SMPRs) for Detection of *F. tularensis* in Aerosol Collection Devices (AOAC SMPR 2016.007)

AOAC SMPR® 2016.007

Standard Method Performance Requirements (SMPRs) for Detection of *Francisella tularensis* in Aerosol Collection Devices

Intended Use: Laboratory or field use by Department of Defense trained operators

1 Applicability

Detection of *Francisella tularensis* in collection buffers from aerosol collection devices. Field-deployable assays are preferred.

2 Analytical Technique

Molecular detection of nucleic acid.

3 Definitions

Acceptable minimum detection level (AMDL).—The predetermined minimum level of an analyte, as specified by an expert committee which must be detected by the candidate method at a specified probability of detection (POD).

Environmental factors.—For the purposes of this SMPR: Any factor in the operating environment of an analytical method, whether abiotic or biotic, that might influence the results of the method.

Exclusivity.—Study involving pure non-target strains, which are potentially cross-reactive, that shall not be detected or enumerated by the candidate method.

Inclusivity.—Study involving pure target strains that shall be detected or enumerated by the candidate method.

Interferents.—A . . . substance in analytical procedures . . . that, at a (the) given concentration, causes a systematic error in the analytical result (International Union of Pure and Applied Chemistry Analytical Chemistry Division Commission on Analytical Reactions and Reagents Definition and Classification of Interferences in Analytical Procedures Prepared for Publication by W.E. Van Der Linden, *Pure & Appl. Chem*. **61**(1), 91–95(1989). Printed in Great Britain, 1989, IUPAC). Sometimes also known as interferants.

Maximum time-to-result.—Maximum time to complete an analysis starting from the collection buffer to assay result.

Probability of detection (POD).—The proportion of positive analytical outcomes for a qualitative method for a given matrix at a specified analyte level or concentration with a ≥0.95 confidence interval.

System false-negative rate.—Proportion of test results that are negative contained within a population of known positives.

System false-positive rate.—Proportion of test results that are positive contained within a population of known negatives.

4 Method Performance Requirements

See Table 1.

Table 1. Method performance requirements

Parameter	Minimum performance requirement
AMDL	2000 standardized cells per mL liquid in the candidate method sample collection buffer
Probability of detection at AMDL within sample collection buffer	≥0.95
Probability of detection at AMDL in environmental matrix materials	≥0.95
System false-negative rate using spiked environmental matrix materials	≤5%
System false-positive rate using environmental matrix materials	≤5%
Inclusivity	All inclusivity strains (Table 3) must test positive at 2x the AMDL[a]
Exclusivity	All exclusivity strains (Table 4 and Annex 1—Part 2) must test negative at 10x the AMDL[a]

[a] 100% correct analyses are expected. All discrepancies are to be retested following the AOAC *Guidelines for Validation of Biological Threat Agent Methods and/or Procedures* [*Official Methods of Analysis of AOAC INTERNATIONAL* (2016) 20th Ed., AOAC INTERNATIONAL, Rockville, MD, USA, Appendix I; http://www.eoma.aoac.org/app_i.pdf].

Table 2. Controls

Control	Description	Implementation
Positive	Designed to demonstrate an appropriate test response. The positive control should be included at a low but easily detectable concentration, and should monitor the performance of the entire assay. The purpose of using a low concentration of positive control is to demonstrate that the assay sensitivity is performing at a previously determined level of sensitivity.	Single use per sample (or sample set) run
Negative	Designed to demonstrate that the assay itself does not produce a detection in the absence of the target organism. The purpose of this control is to rule out causes of false positives, such as contamination in the assay or test.	Single use per sample (or sample set) run
Inhibition	Designed to specifically address the impact of a sample or sample matrix on the assay's ability to detect the target organism.	Single use per sample (or sample set) run

© 2016 AOAC INTERNATIONAL

Table 3. Inclusivity panel				
No.	UCC[a] ID	Genus and species	Strain	Characteristics
1	FRAN001	*Francisella tularensis*	subsp. *tularensis*	Type A2 (Type strain)
2	FRAN004	*Francisella tularensis*	subsp. *holarctica* (LVS)	Type B (Russian)
3	FRAN012	*Francisella tularensis*	subsp. *holarctica*	Type B (United States)
4	FRAN016	*Francisella tularensis*	subsp. *tularensis* (SCHU S4)	Type A1 (United States)
5	FRAN024	*Francisella tularemia*	subsp. *holarctica* JAP (Cincinnati)	Type B (Japanese)
6	FRAN025	*Francisella tularensis*	subsp. *tularensis* (VT68)	Type A1 (United States)
7	FRAN029	*Francisella tularensis*	subsp. *holarctica* (425)	Type B (United States)
8	FRAN031	*Francisella tularensis*	subsp. *tularensis* (Scherm)	Type A1 (United States)
9	FRAN072	*Francisella tularensis*	subsp. *tularensis* (WY96)	Type A2 (United States)
10	N/A	*Francisella tularensis*	subsp. *mediasiatica*	

[a] UCC = Department of Defense Unified Culture Collection; components available through Biodefense and Emerging Infections Research Resources Repository.

Table 4. Exclusivity panel (near-neighbor)		
No.	Species	Strain
1	*Francisella philomiragia*	Jensen O#319L ATCC 25015
2	*Francisella philomiragia*	Jensen O#319-029 ATCC 25016
3	*Francisella philomiragia*	Jensen O#319-036 ATCC 25017
4	*Francisella philomiragia*	Jensen O#319-067 ATCC 25018
5	*Francisella philomiragia*	D7533, GA012794
6	*Francisella philomiragia*	E9923, GA012801
7	*Francisella novicida*	D9876, GA993548
8	*Francisella novicida*	F6168, GA993549
9	*Francisella novicida*	U112, GA993550
10	*Francisella hispaniensis*	DSM 22475

5 System Suitability Tests and/or Analytical Quality Control

The controls listed in Table 2 shall be embedded in assays as appropriate. Manufacturer must provide written justification if controls are not embedded in the assay.

6 Validation Guidance

AOAC INTERNATIONAL Methods Committee Guidelines for Validation of Biological Threat Agent Methods and/or Procedures [*Official Methods of Analysis AOAC INTERNATIONAL* (2016) Appendix I].

Inclusivity and exclusivity panel organisms used for evaluation must be characterized and documented to truly be the species and strains they are purported to be.

In silico analysis.—In silico screening shall be performed on signature sequences (e.g., oligo primers/probes/amplicons) to predict specificity and inclusivity across available sequenced *Francisella* strains. In silico results are suggestive of potential performance issues. Basic Local Alignment Search Tool (BLAST) (or a comparable tool) should be used to examine potential hybridization events between signature components and available *Francisella* genomic sequence data in GenBank®. Results of in silico analyses shall be included in method/assay performance evaluation reports.

7 Maximum Time-to-Results

Within 4 h.

8 Guidance on Combining DNA for Exclusivity Evaluation

Organisms may be tested as isolated DNA, or combined to form a pool of isolated DNA. Isolated DNA may be combined into pools of up to 10 exclusivity panel organisms, with each panel organism represented at 10 times the AMDL, where possible. If an unexpected result occurs, each of the exclusivity organisms from a failed pool must be individually retested at 10 times the AMDL.

Approved by the AOAC Stakeholder Panel on Agent Detection Assays (SPADA). Final Version Date: March 22, 2016.

© 2016 AOAC INTERNATIONAL

Annex 1. Environmental Factors for Validating Biological Threat Agent Detection Assays

[Adapted from the Environmental Factors Panel approved by SPADA on June 10, 2010.]

The Environmental Factors Studies supplement the biological threat agent near-neighbor exclusivity testing panel. There are three parts to Environmental Factors Studies: Part 1—Environmental matrix samples; Part 2—Environmental organisms study; and Part 3—Potential interferents applicable to Department of Defense applications (added in June 2015 for the Department of Defense project).

Part 1: Environmental Matrix Samples—Aerosol Environmental Matrices

Method developers shall obtain environmental matrix samples that are representative and consistent with the collection method that is anticipated to ultimately be used in the field. This includes considerations that may be encountered when the collection system is deployed operationally such as collection medium, duration of collection, diversity of geographical areas that will be sampled, climatic/environmental conditions that may be encountered and seasonal changes in the regions of deployment.

Justifications for the selected conditions that were used to generate the environmental matrix and limitations of the validation based on those criteria must be documented.

- Method developers shall test the environmental matrix samples for interference using samples inoculated with a target biological threat agent sufficient to achieve 95% probability of detection.

- Cross-reactivity testing will include sufficient samples and replicates to ensure each environmental condition is adequately represented.

Part 2: Environmental Panel Organisms

This list is comprised of identified organisms from the environment.

Inclusion of all environmental panel organisms is not a requirement if a method developer provides appropriate justification that the intended use of the assay permits the exclusion of specific panel organisms. Justification for exclusion of any environmental panel organism(s) must be documented and submitted.

Organisms and cell lines may be tested as isolated DNA, or as pools of isolated DNA. Isolated DNA may be combined into pools of up to 10 panel organisms, with each panel organism represented at 10 times the AMDL, where possible. The combined DNA pools are tested in the presence (at 2 times the AMDL) and absence of the target gene or gene fragment. If an unexpected result occurs, each of the individual environmental organisms from a failed pool must be individually retested at 10 times the AMDL with and without the target gene or gene fragment at 2 times the AMDL in the candidate method DNA elution buffer.

DNA in this list that already appear in the inclusivity or exclusivity panel do not need to be tested again as part of the environmental factors panel.

- **Potential bacterial biothreat agents**
  Bacillus anthracis Ames
  Yersinia pestis Colorado-92
  Francisella tularensis subsp. *tularensis* Schu-S4
  Burkholderia pseudomallei
  Burkholderia mallei
  Brucella melitensis

- **Cultivatable bacteria identified as being present in air, soil, or water**
  Acinetobacter lwoffii
  Agrobacterium tumefaciens
  Bacillus amyloliquefaciens
  Bacillus cohnii
  Bacillus psychrosaccharolyticus
  Bacillus benzoevorans
  Bacillus megaterium
  Bacillus horikoshii
  Bacillus macroides
  Bacteroides fragilis
  Burkholderia cepacia
  Burkholderia gladoli
  Burkholderia stabilis
  Burkholderia plantarii
  Chryseobacterium indologenes
  Clostridium sardiniense
  Clostridium perfringens
  Deinococcus radiodurans
  Delftia acidovorans
  Escherichia coli K12
  Fusobacterium nucleatum
  Lactobacillus plantarum
  Legionella pneumophilas
  Listeria monocytogenes
  Moraxella nonliquefaciens
  Mycobacterium smegmatis
  Neisseria lactamica
  Pseudomonas aeruginosa
  Rhodobacter sphaeroides
  Riemerella anatipestifer
  Shewanella oneidensis
  Staphylococcus aureus
  Stenotophomonas maltophilia
  Streptococcus pneumoniae
  Streptomyces coelicolor
  Synechocystis
  Vibrio cholerae

- **Microbial eukaryotes**
  Freshwater amoebae:
  - *Acanthamoeba castellanii*
  - *Naegleria fowleri*

  Fungi:
  - *Alternaria alternata*
  - *Aspergillus fumagatis*
  - *Aureobasidium pullulans*
  - *Cladosporium cladosporioides*
  - *Cladosporium sphaerospermum*
  - *Epicoccum nigrum*
  - *Eurotium amstelodami*
  - *Mucor racemosus*
  - *Paecilomyces variotii*
  - *Penicillum chrysogenum*
  - *Wallemia sebi*

© 2016 AOAC INTERNATIONAL

- **DNA from higher eukaryotes**

  Plant pollen (if pollen is unavailable, vegetative DNA is acceptable):

  Zea mays (corn)
  Pinus spp. (pine)
  Gossypium spp. (cotton)

  Arthropods:

  Aedes aegypti (ATCC/CCL-125(tm) mosquito cell line)
  Aedes albopictus (Mosquito C6/36 cell line)
  Dermatophagoides pteronyssinus
  (Dust mite-commercial source)
  Xenopsylla cheopis Flea (Rocky Mountain Labs)
  Drosophilia cell line
  Musca domestica
  (housefly; ARS, USDA, Fargo, ND, USA)
  Gypsy moth cell lines
  [LED652Y cell line (baculovirus); Invitrogen]
  Cockroach (commercial source)
  Tick (*Amblyomma* and *Dermacentor* tick species for *F. tularensis* detection assays) (added by SPADA on March 22, 2016)

  Vertebrates:

  Mus musculus (ATCC/HB-123) mouse
  Rattus norvegicus (ATCC/CRL-1896) rat
  Canis familiaris(ATCC/CCL-183) dog
  Felis catus (ATCC/CRL-8727) cat
  Homo sapiens (HeLa cell line ATCC/CCL-2) human
  Gallus gallus domesticus (chicken)
  Capri hirca (goat) (added by SPADA on September 1, 2015)

- **Biological insecticides**

Strains of *B. thuringiensis* present in commercially available insecticides have been extensively used in hoaxes and are likely to be harvested in air collectors. For these reasons, it should be used to assess the specificity of these threat assays.

B. thuringiensis subsp. *israelensis*
B. thuringiensis subsp. *kurstaki*
B. thuringiensis subsp. *morrisoni*
Serenade (Fungicide) *B. subtilis* (QST713)

Viral agents have also been used for insect control. Two representative products are:

Gypcheck for gypsy moths (*Lymanteria dispar* nuclear polyhedrosis virus)
Cyd-X for coddling moths (Coddling moth granulosis virus)

Part 3: Potential Interferents Study

The Potential Interferents Study supplements the Environmental Factors Study, and is applicable to all biological threat agent detection assays for Department of Defense applications. Table 5 provides a list of potential interferents that are likely to be encountered in various Department of Defense applications.

Method developers and evaluators shall determine the most appropriate potential interferents for their application. Interferents shall be spiked at a final test concentration of 1 µg/mL directly into the sample collection buffer. Sample collection buffers spiked with potential interferents shall by inoculated at 2 times the AMDL (or acceptable minimum identification level; AMIL) with one of the target biological threat agents.

Spiked/inoculated sample collection buffers shall be tested using the procedure specified by the candidate method. A candidate method that fails at the 1 µg/mL level may be reevaluated at lower concentrations until the inhibition level is determined.

It is expected that all samples are correctly identified as positive.

Table 5 is offered for guidance and there are no mandatory minimum requirements for the number of potential interferents to be tested.

© 2016 AOAC INTERNATIONAL

Table 5. Potential interferents

Compound		Potential theaters of operation
Group 1: Petroleum-based	JP-8[a]	Airfield
	JP-5[b]	Naval
	Diesel/gasoline mixture	Ground
	Fog oil (standard grade fuel number 2)	Naval, ground
	Burning rubber[c]	Ground, airfield
Group 2: Exhaust	Gasoline exhaust	Ground
	Jet exhaust	Naval, airfield
	Diesel exhaust	Ground
Group 3: Obscurants	Terephthalic acid[d]	Ground
	Zinc chloride smoke[e]	Ground
	Solvent yellow 33[f]	Ground
Group 4: Environmental	Burning vegetation	Ground, airfield
	Road dust	Ground
	Sea water (sea spray)	Naval
Group 5: Chemicals	Brake fluid[g]	All
	Brake dust[h]	Ground
	Cleaning solvent, *MIL-L-63460*[i]	All
	Explosive residues: High explosives[j] Artillery propellant[k]	All

[a] JP-8: Air Force formulation jet fuel.

[b] JP-5: A yellow kerosene-based jet fuel with a lower flash point developed for use in aircraft stationed aboard aircraft carriers, where the risk from fire is particularly great. JP-5 is a complex mixture of hydrocarbons, containing alkanes, naphthenes, and aromatic hydrocarbons.

[c] Burning rubber (tire smoke): Gaseous C1-C5 hydrocarbons: methane; ethane; isopropene; butadiene; propane. Polycyclic aromatic hydrocarbons (58–6800 ng/m^3): parabenzo(*a*)pyrene; polychlorinated dibenzo-*p*-dioxins (PCDD); polychlorinated dibenzofurans (PCDF). Metals (0.7–8 mg/m^3): zinc; lead; cadmium.

[d] Terephthalic acid: Used in the AN/M83 hand grenade currently used by US military.

[e] Zinc chloride smoke: Also known as “zinc chloride smoke” and “HC smoke.” Was used in the M8 grenade and still used in 155 mm artillery shells. HC smoke is composed of 45% hexachloroethane, 45% zinc oxide, and 10% aluminum.

[f] Solvent yellow 33 [IUPAC name: 2-(2-quinolyl)-1,3-indandione] is a new formulation being develop for the M18 grenade.

[g] Brake fluid: DOT 4 is the most common brake fluid, primarily composed of glycol and borate esters. DOT 5 is silicone-based brake fluid. The main difference is that DOT 4 is hydroscopic whereas DOT 5 is hydrophobic. DOT 5 is often used in military vehicles because it is more stable over time requires less maintenance.

[h] Brake dust: Fe particles caused by abrasion of the cast iron brake rotor by the pad and secondly fibers from the semi metallic elements of the brake pad. The remainder of the dust residue is carbon content within the brake pad.

[i] MIL-L-63460, “Military Specification, Lubricant, Cleaner and Preservative for Weapons and Weapons Systems;” trade name “Break-Free CLP” (http://www.midwayusa.com/product/1106170293/break-free-clp-bore-cleaning-solvent-lubricant-rust-preventative-liquid).

[j] High explosives: The M795 155 mm projectile is the U.S. Army/Marine Corp’s current standard projectile containing 10.8 kg TNT. The M795 projectile replaced the M107 projectile that contained Composition B, which is a 60/40 mixture of RDX/TNT. RDX is cyclotrimethylene trinitramine. Suggestion: Test RDX/TNT together.

[k] Artillery propellant: Modern gun propellants are divided into three classes: single-base propellants, which are mainly or entirely nitrocellulose based; double-base propellants composed of a combination of nitrocellulose and nitroglycerin; and triple base composed of a combination of nitrocellulose and nitroglycerin and nitroguanidine. Suggestion: Test total nitrocellulose/nitroglycerin nitroguanidine together.

© 2016 AOAC INTERNATIONAL

Annex V

Screening of mosquitoes as vectors of *Francisella tularensis* in Portugal

SCREENING OF MOSQUITOES AS VECTORS OF *FRANCISELLA TULARENSIS* IN PORTUGAL

CARINA LUÍSA CARVALHO [1], LÍBIA ZÉ-ZÉ [1], ELSA LECLERC DUARTE[2], MARIA SOFIA NÚNCIO[1], ISABEL LOPES DE CARVALHO[1]

[1]Center for Vectors and Infectious Diseases Research, National Institute of Health Dr. Ricardo Jorge, Águas de Moura, Portugal,

[2]ICAAM, University of Évora, Portugal

Tularemia is a zoonosis caused by *Francisella tularensis* that has recently emerged in new locations, populations and settings (1). This contagious septicemic disease affects mainly hares, sylvatic rabbits, rats, mice and other rodents. In some circumstances, the disease can also affect humans, domestic animals (herbivores and small carnivores), birds, fish and amphibians. The major route of infection is the skin by direct contact with dead or infected animals. Other routes of infection are the eye conjunctiva, mouth and nose mucous membrane (drinking contaminated water, ingestion of meat from sick animals or inhalation) or arthropod bites (2). The most important pathogenic subspecies are *F. tularensis* subsp. *holarctica* that occurs throughout the Northern hemisphere and *F. tularensis* subsp. *tularensis* that occurs usually in North America. Mosquitoes from genera *Culex* and *Aedes* are considered important vectors for *F. tularensis,* especially in Sweden (3). In Portugal, there are 40 species of mosquitoes reported, being *Ochlerotatus caspius*, *Culex pipiens* (Figure 1) and *Cx. theileri* the most frequent (4). *F. tularensis* subsp. *holarctica* was already detected in *Dermacentor reticulatus* ticks (1), however the role of mosquitoes remains unknown.

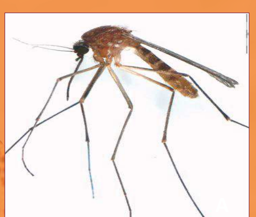


Figure 1. *Culex pipiens* (**A**) *and Ochlerotatus caspius* (**B**), courtesy of Hugo Osório, Center for Vectores and Infectious Diseases Research.

In this work, the role of mosquitoes in the transmission of *F. tularensis* in Portugal was investigated. We aimed to clarify if the species of mosquitoes reported in Portugal could act as competent vectors for *F. tularensis,* as reported in some European countries.

An ongoing epidemiologic surveillance program on arthropod vectors (REVIVE) provided the samples that were analyzed in this study. A total of 4949 mosquitoes were investigated for the presence of *F. tularensis;* of which 1373 (68 pools) were captured during the year of 2011, 143 specimens were captured between 2007 and 2010, all over the national territory and 3433 mosquitoes (80 pools) were captured during the year of 2007 in the region of Algarve. The mosquitoes of this last group were collected in same year of the last outbreak in Spain. Pool mosquito samples were extracted using phenol:chloroform. Individual specimens DNA was extracted using DNeasy Blood and Tissue kit (Qiagen). A nested PCR for specific partial amplification of *tul4* gene was used for *F. tularensis* nucleic acid detection, as described by Karhukorpi and Karhukorpi (2001) (5).

Table 1: Mosquitoes studied for the presence of *F. tularensis.* (*) A small number of *Aedes aegypti* females from Madeira island were also analyzed.

Mosquito Genera	Mosquito Species	Number of Mosquitoes analyzed	Genera percentage (%)
Culex	*Cx. pipiens*	2352	63.97
	Cx. theileri	645	
	Cx. perexiguus	78	
	Cx. hortensis	5	
	Cx. univitattus	86	
Ochlerotatus	*Oc. caspius*	1692	35.34
	Oc. detritus	57	
Anopheles	*An maculipennis*	8	0.42
	An atroparvus	13	
Culiseta	*Cs. longiareolata*	5	0.14
	Cs. annulata	2	
Aedes (*)	*A. aegypti*	6	0.12

All samples investigated were negative for the presence of *F. tularensis* (Table 1). These results suggest that in Portugal mosquitoes do not play a crucial role as vectors for *F. tularensis.* Ticks are probably the most important vectors for this pathogen as it happens in the majority of countries were tularemia is endemic.

CLC a is PhD student in Veterinary Medicine with a fellowship from Fundação para a Ciência e a Tecnologia (FCT) - SFRH /BD/79225/2011.
This study was partially supported by PTDC/SAU – ESA/104947/2008 project, FCT. We acknowledge Dr. Hugo Osório for the cession of DNA samples from female mosquitoes and REVIVE project (Rede de Vigilância de Vectores), Center for Vectors and Infectious Diseases, for the remaining mosquito samples.

(1) de Carvalho IL, Escudero R, Garcia-Amil C, Falcão H, Anda P, Núncio MS. *Francisella tularensis,* Portugal. Emerg Infect Dis. 2007 Apr 13(4):666-7.
(2) de Carvalho IL, Núncio MS, David de Morais J. Tularémia. Acta Med Port. 2009 May-Jun 22(3):281-90.
(3) Lundström JO, Andersson AC, Bäckman S, Schäfer ML, Forsman M, Thelus J. Transstadial transmission of *Francisella tularensis holarctica* in mosquitoes, Sweden. Emerg Infect Dis. 2011 May 17(5):794-9.
(4) Osório HC, Amaro F, Zé-Zé L, Pardal S, Mendes L, Ventim R, Ramos JA, Nunes S, REVIVE workgroup, Alves MJ. Mosquito species distribution in mainland Portugal 2005-2008. Eur Mosq Bull.2010 Oct:187-193
(5) Karhukorpi EK, Karhukorpi J. Rapid laboratory diagnosis of ulceroglandular tularemia with polymerase chain reaction. Scand J InfectDis 2001; 33:382-385

7th International Conference on tularemia, Breckenridge, Colorado Sept 17-20, 2012

Annex VI

Accession numbers (GenBank) of the sequences produced during this work

1. Rabbit haemorrhagic disease virus 2 (RHDV2)

Region of Portugal	Year of collection	Accession numbers	
		Released	Unreleased
Mainland	2014	KU665600-601	KY454641-654
	2015	-	KY454655-662
	2016	Kx132812-813	KY454663-668
Azores archipelago	2015	KP862922-33	KY454669-687
Madeira archipelago	2016	Ky310747-749	
	2017	Ky783700-703	
Berlengas archipelgo	2016	Ky247124-125	

2. *Francisella* tularensis and *Francisella*-like endosymbionts

Organism	Host	Year of collection	Accession numbers	
			Released	Unreleased
Francisella tularensis	Lagomorphs	2011	KJ477081	-
***Francisella*-like endosymbionts**	*Dermacentor reticulatus*	2014		MF497789-794
	Hyalomma marginatum	2012		MF497787
	Hyalomma lusitanicum	2012		MF497788