Insights into the host-pathogen interactions: *Lagovirus* in leporids

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It would be possible to describe everything scientifically, but it would make no sense. It would be a description without meaning, as if you described a Beethoven symphony as a variation of wave pressure.

Albert Eistein

Foreword

In compliance with the no. 2 of article 4 of the General Regulation of Third Cycles of the University of Porto and with the article 31 of the Decree-Law no. 74/2006, of 24 March, with the alteration introduced by the Decree-Law no. 230/2009, of 14 September, the results of already published works were totally used and included in some of the chapters of this dissertation. As these works were performed in collaboration with other authors, the candidate clarifies that, in all these works, participated in obtaining, interpreting, analyzing and discussing the results, as well as in the writing of the published forms.

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Summary

The family Leporidae belongs to the order Lagomorpha and has 11 extant genera distributed worldwide. In Europe, two genera naturally occur: *Lepus*, originating from North America, and *Oryctolagus*, whose place of origin is Iberian Peninsula. *Oryctolagus* has a single extant taxon, the European rabbit (*Oryctolagus cuniculus*) and *Lepus* has six taxa inhabiting Europe, the most widespread being the European brown hare (*Lepus europaeus*). *O. cuniculus* and *L. europaeus* populations suffered severe declines in Europe. Along with other factors, the viral diseases rabbit hemorrhagic disease (RHD), affecting European rabbits, and European brown hare syndrome (EBHS), affecting hares, are responsible for these declines.

The causative agents of RHD and EBHS are the rabbit hemorrhagic disease virus (RHDV) and the European brown hare syndrome virus (EBHSV), respectively. RHDV and EBHSV are single-stranded, positive-sense RNA viruses with ~ 7.4 kb in length, classified as *Lagovirus* from the family *Caliciviridae*. For RHDV, several genogroups (G1-G6) were identified, as well as non-pathogenic forms. The information available for the Iberian Peninsula until 2011 indicated the circulation of G1 in wild populations. In 2010, outbreaks of RHD with unique features were recorded in France, and a new RHDV variant was identified. The emergence of this new RHDV variant, related to, but highly distinct from the genogroups previously identified, triggered the study of the new RHDV variant within the scope of the host-pathogen interactions of lagoviruses.

The first cases of the new RHDV variant in Portugal mainland date back to late 2012 in the north and early 2013 in the south. Analysis of a partial fragment of the capsid protein gene revealed 99 % of nucleotide identity with a strain of the new variant previously identified in Spain. Phylogenetic analysis positioned the new variant in an independent cluster in between non-pathogenic rabbit caliciviruses. In the following years, samples from rabbits found dead in the field were collected and the screening of these samples revealed a replacement of the former circulating G1 by the new RHDV variant. A selective advantage of the new RHDV variant over other genogroups, possibly by overcoming the existing immunity against older strains, might be at the origin of this replacement.

The evolution of RNA viruses and the generation of genetic variability is often associated with recombination. Recombination might assist the emergence of new pathogenic viruses and the increase of pathogenic potential and virulence in pre-existing strains. Recombination was previously described for RHDV with recombination breakpoints located in several regions of the genome, including within the polymerase and the capsid. A striking pattern of recombination between non-structural and structural proteins was recovered for Iberian strains identified as new RHDV variant. While for the structural proteins these strains belonged to the new RHDV variant cluster, for the non-structural backbone they clustered either with non-pathogenic rabbit caliciviruses or with the pathogenic G1 strains. This suggested at least two independent recombination events. These results further imply the presence of non-pathogenic rabbit caliciviruses in the Iberian Peninsula that remain undetected. The G1 recombinant was later detected in Azores, a Portuguese archipelago in the middle of the Atlantic Ocean. This likely man-mediated introduction caused severe damage in Azorean rabbit populations and replaced the unique G3-G5 *like* strains that circulated in the archipelago before the arrival of the new RHDV variant.

Knowledge on EBHSV lags behind that on RHDV. To overcome this and better understand the evolution of EBHSV, full-genome sequences were obtained for two samples collected in the early 1980s in Sweden, the site of original diagnosis. These correspond to the oldest sequences of EBHSV and added information to the few available sequences. The genomic organization of these samples is similar to that reported for a more recent French strain, and most of the cleavage sites were conserved. As expected, most amino acid differences were located in the VP60 protein, confirming the exposed nature of this protein.

Evolution of EBHSV was further characterized with a molecular study using samples collected in Sweden, spanning the first years of outbreaks until 2008. The molecular clock dating reinforces the hypothesis that EBHSV circulation predates its emergence. Phylogenetic analysis including other European strains revealed two EBHSV lineages, one that disappeared in Sweden and another more recent that was able to persist and spread within Europe. Signatures of positive selection in codons located in exposed regions of the capsid and antigenic differences between lineages indicated a possible role for immune-mediated selection.

The first interactions of the virus with the surface of host cells determine viral tropism and are mediated by host molecules acting as attachment factors. Previous works identified histo-blood group antigens (HBGAs) as attachment factors for RHDV.

These carbohydrates are present on the epithelial cells of the upper respiratory and digestive tracts, where the virus first contacts with the host. Although other receptors are likely involved in the process of virus entry to the cell, this was an important first step towards unraveling the RHDV infectious process. The phylogenetic proximity of RHDV and EBHSV might foresee the same pathways of virus entry, thus the binding of EBHSV to glycans was tested. Our findings showed that EBHSV seems to use GlcNAc-terminated O-glycans to establish the first contact with the cell, which represents a distinct feature comparing to RHDV that might be central for the EBHSV infectious cycle.

Lagoviruses are considered specialist viruses, affecting only their natural hosts. However, the new RHDV variant has challenged this view and several episodes of host jumps have been described. Additionally, we reported a spillover event in the *Lagovirus* genus, corresponding to the earliest evidence of RHDV affecting a species other than the European rabbit. Investigation of two hare samples, collected in the Iberian Peninsula in the 1990s, with clinical findings consistent with a *Lagovirus* infection at necropsy, revealed two independent RHDV host jumps. Phylogenetic analysis further confirmed that G1 strains were responsible for both cases and analysis of nuclear and mitochondrial markers confirmed the species as *L. granatensis*. The mechanisms underlying the ability of RHDV to host switch are not yet fully understood.

RHDV and EBHSV emerged in the 1980s, but their origins are still uncertain. There are several indications of a former presence in Europe, such as the existence of antibodies against both viruses in samples collected before their emergence. These data suggest an evolution from a non-pathogenic form that, for reasons yet uncovered, acquired virulence. Another hypothesis for RHDV and EBHSV emergence is a species jump from *Sylvilagus floridanus*. This is consistent with the introduction of this species in Europe a few years before the emergence of these viruses. Since host jumps are relatively frequent in the evolution of single-stranded RNA viruses, are more likely between phylogenetically-related hosts, and the adaptation to a novel host might be assisted by recombination, we investigated possible host jumps in the *Lagovirus* genus.

A comprehensive study on host binding of non-pathogenic viruses and the new RHDV variant was performed. Their binding to *Oryctolagus*, *Lepus* and *Sylvilagus* tissues of the upper respiratory and digestive tracts was similar to that already observed for the classical RHDV. The new RHDV variant and the non-pathogenic strains tested also use HBGAs to attach to host cells, but a strong binding affinity to heparin was also observed. The affinity of each strain to HBGAs varies and might be related with adaptation and co-evolution with the host.

Sumário

A família Leporidae pertence à ordem Lagomorpha e é composta por 11 géneros distribuídos por todo o mundo, dois dos quais ocorrem naturalmente na Europa: o género *Lepus*, originário da América do Norte, e o género *Oryctolagus*, cuja origem está na Península Ibérica. Este último tem um único *taxon*, o coelho-bravo (*Oryctolagus cuniculus*), enquanto o género *Lepus* tem seis *taxa* presentes na Europa, com a lebre parda (*Lepus europaeus*) a ocupar a maior área geográfica. As populações destas duas espécies viram os seus efetivos reduzirem-se nas últimas décadas na Europa. A doença hemorrágica viral (DHV) e a síndrome da lebre parda (SLP), duas doenças de origem viral que afetam o coelho-bravo e a lebre parda, respetivamente, são dois fatores importantes no declínio destas populações.

Os agentes responsáveis pela DHV e pela SLP são respetivamente o vírus da doença hemorrágica viral (VDHV) e o vírus da síndrome da lebre parda (VSLP). O VDHV e o VSLP são vírus de cadeia simples, de sentido positivo, cujo material genético é ARN e com ~ 7.4 kb, classificados como *Lagovirus* da família *Caliciviridae*. Para o VDHV foram identificados vários genogrupos (G1-G6), assim como formas não-patogénicas. Até 2011, a informação disponível para a Península Ibérica indicava uma circulação de G1 em populações selvagens. Em 2010, surgiram em França surtos de DHV com características únicas, levando à identificação de uma nova variante, relacionada mas altamente distinta dos genogrupos previamente identificados. O seu aparecimento originou o estudo da nova variante no âmbito das interações patogénio-hospedeiro dos lagovírus.

Os primeiros casos da nova variante da DHV em Portugal continental datam de 2012 no norte e início de 2013 no sul. A análise de um fragmento do gene que codifica a proteína da cápside revelou 99 % de identidade nucleotídica com uma estirpe da nova variante previamente identificada em Espanha. A análise filogenética posicionou a nova variante num grupo independente, entre as formas não-patogénicas do VDHV. Nos anos seguintes, a recolha e análise de amostras de coelhos encontrados mortos no campo revelou uma substituição da estirpe circulante, G1, pela nova variante. Uma vantagem seletiva da nova variante sobre os outros genogrupos pode estar na origem desta

substituição, possivelmente por conseguir ultrapassar a imunidade desenvolvida contra as estirpes anteriores.

A evolução dos vírus de ARN e o aumento de variabilidade genética pode estar associada à recombinação. Este fenómeno facilita o aparecimento de novos vírus e o aumento do potencial patogénico e da virulência de estirpes pré-existentes. Para o VDHV foram previamente descritos eventos de recombinação, cujos pontos de recombinação estão localizados em várias regiões do genoma, incluindo nos genes da polimerase e da proteína da cápside. Em estirpes ibéricas identificadas como pertencentes à nova variante da DHV foi encontrado um padrão de recombinação entre proteínas não-estruturais e proteínas estruturais. Enquanto para as proteínas estruturais estas estirpes pertenciam ao grupo da nova variante, para as proteínas não-estruturais estas estirpes agrupavam com formas não-patogénicas ou com estirpes G1. Estes resultados sugerem pelo menos dois eventos de recombinação independentes e implicam ainda a presença de formas não-patogénicas na Península Ibérica. O recombinante G1/nova variante foi mais tarde detetado nos Açores. A introdução da nova variante neste arquipélago, com uma origem provavelmente antropogénica, causou declínios severos nas populações e levou a uma aparente substituição das estirpes G3-G5 *like* que circulavam no arquipélago anteriormente.

O conhecimento acerca do VSLP é substancialmente menor que o do VDHV. Para colmatar este desconhecimento e melhor compreender a evolução do VSLP, foram obtidas sequências completas de duas amostras recolhidas em 1982 na Suécia, o primeiro país onde foi descrita a doença, sendo por isso as sequências mais antigas disponíveis para o VSLP. A organização do genoma destas amostras é semelhante à observada para uma estirpe francesa mais recente, e a maioria dos locais de clivagem no genoma são conservados. Tal como esperado, a maioria das diferenças aminoacídicas estão localizadas na proteína da cápside, região do vírus que está mais exposta a uma resposta imunitária do hospedeiro.

A evolução do VSLP foi ainda estudada em amostras recolhidas na Suécia desde os primeiros surtos até 2008. A calibração do relógio molecular reforçou a hipótese de uma circulação do vírus que antecede a sua emergência. A análise filogenética, que incluiu outras estirpes europeias, revelou duas linhagens do VSLP: uma específica das amostras da Suécia e que terá desaparecido, e outra, mais recente, que persistiu e se difundiu nos restantes países europeus. Os sinais de seleção positiva em codões localizados em regiões expostas da cápside e as diferenças antigénicas entre linhagens sugerem a existência de uma pressão seletiva exercida pelo sistema imunitário.

As primeiras interações do vírus com a superfície das células do hospedeiro determinam o tropismo viral e são mediadas por moléculas do hospedeiro que atuam como fatores de ligação. Trabalhos anteriores identificaram os antigénios do grupo sanguíneo ABH como fatores de ligação para o VDHV. Estes carbohidratos estão presentes nas células epiteliais do aparelho respiratório superior e do aparelho digestivo, onde o vírus estabelece o primeiro contacto com o hospedeiro. Embora outros recetores possam estar envolvidos no processo de entrada do vírus, esta informação ajudou na compreensão do processo infecioso do VDHV. A proximidade filogenética do VDHV e do VSLP fazem prever modos de entrada na célula semelhantes; assim, foi testada a ligação do VSLP aos referidos carbohidratos. Os nossos resultados mostraram que, no estabelecimento do primeiro contacto com a célula, o VSLP parece preferir carbohidratos O-glicosilados com uma cadeia terminal de N-acetilglicosamina. Este padrão de ligação ao hospedeiro constitui uma diferença relativamente ao VDHV que pode ser central no processo infecioso do VSLP.

Os lagovírus são considerados vírus especialistas, isto é, que afetam apenas os seus hospedeiros naturais. A nova variante tem desafiado esta visão, uma vez que têm sido descritos vários episódios de *species jumps* para esta variante. Adicionalmente, foi possível observar a presença de G1 em lebres ibéricas, o que corresponde à evidência mais antiga de um lagovírus a infetar outra espécie que não o seu hospedeiro natural. A confirmação dos animais como lebres ibéricas foi realizada através de marcadores nucleares e mitocondriais. A análise de duas amostras de lebre ibérica, recolhidas na Península Ibérica nos anos 1990 com sinais clínicos consistentes com uma infeção por um lagovírus, permitiram verificar dois eventos independentes. A análise filogenética confirmou que diferentes estirpes G1 estiveram na origem destes casos. Os mecanismos que permitiram ao VDHV transitar de uma espécie para outra não são ainda totalmente compreendidos.

O VDHV e o VSLP emergiram na década de 1980, mas as suas origens permanecem incertas. Existem várias indicações de uma presença anterior na Europa, como por exemplo a existência de anticorpos contra ambos os vírus em amostras recolhidas antes das datas aceites como início dos surtos destas doenças. Estes resultados sugerem uma evolução a partir de uma forma não-patogénica que, por razões ainda não totalmente esclarecidas, adquiriu virulência. Outra hipótese para explicar o aparecimento destes vírus é um *species jump* a partir de *Sylvilagus floridanus*. A introdução desta espécie na Europa alguns anos antes do aparecimento do VDHV e do VSLP reforça esta hipótese. A investigação da ocorrência de *species jumps* em lagovírus foi conduzida tendo em conta que estes fenómenos são relativamente

frequentes na evolução dos vírus de ARN de cadeia simples e entre hospedeiros filogeneticamente próximos, e ainda que a adaptação a um novo hospedeiro pode ser facilitada pela existência de recombinação.

Tendo em conta as hipóteses acima mencionadas de aparecimento dos lagovírus, estudou-se a ligação ao hospedeiro de formas não-patogénicas e da nova variante da DHV. A sua ligação a tecidos dos aparelhos respiratório superior e digestivo de *Oryctolagus*, *Lepus* e *Sylvilagus* é semelhante à já observada para a variante clássica da DHV. A nova variante e as estirpes não-patogénicas testadas usam igualmente antigénios do grupo sanguíneo ABH para se ligar às células do hospedeiro. No entanto, foi também observada uma grande afinidade com a heparina. A afinidade de cada estirpe para os antigénios do grupo sanguíneo ABH varia e pode estar relacionada com a adaptação e co-evolução com o hospedeiro.

Keywords

- Rabbit hemorrhagic disease virus
- New variant of rabbit hemorrhagic disease virus
- Iberian Peninsula
- Recombination
- European brown hare syndrome virus
- Viral evolution
- Leporids
- Host-virus interaction
- Attachment factors
- Species jump

Palavras-chave

- Vírus da doença hemorrágica viral
- Nova variante do vírus da doença hemorrágica viral
- Península Ibérica
- Recombinação
- Vírus da síndrome da lebre parda
- Evolução viral
- Leporídeos
- Interações vírus-hospedeiro
- Fatores de ligação
- Transição de vírus entre espécies

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Abbreviations

AI: association index
ATP: adenosine triphosphate
ATPase: adenosine triphosphatase
bp: base pairs
BSA: bovine serum albumin
CAR: coxsackievirus and adenovirus receptor
CCR5: C-C motif chemokine receptor 5
CD4: cluster of differentiation 4
cDNA: complementary deoxyribonucleic acid
CDV: canine distemper virus
CPV: canine parvovirus
CXCR4: C-X-C motif chemokine receptor type 4
DC-SIGN: dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
DIC: disseminated intravascular coagulation
DNA: deoxyribonucleic acid
dNTP: deoxyribose nucleotide triphosphate
dpi: days post-infection
EBHS: European brown hare syndrome
EBHSV: European brown hare syndrome virus
ELISA: enzyme-linked immunosorbent assay
FCV: feline calicivirus
FEL: fixed effects likelihood
FPV: feline panleukopenia virus
FUBAR: fast unbiased Bayesian approximation
GAG: glycosaminoglycan
GlcNAc: *N*-acetylglucosamine
GTR: general time-reversible
H&E: hematoxylin and eosin
HBGAs: histo-blood group antigens
HIV: human immunodeficiency virus

HPD: highest density probability

HRP: horseradish peroxidase

HSPG: heparan sulfate proteoglycan

IgA: immunoglobulin A

IgG: immunoglobulin G

IGHG: immunoglobulin gamma heavy chain

IgM: immunoglobulin M

IHC: immunohistochemistry

IP: Iberian Peninsula

JAM-1: junctional adhesion molecule 1

kb: kilobases

LDLR: low density lipoprotein receptor

MAA: *Maackia amurensis* agglutinin

MAb: monoclonal antibody

MC: maximum clade

MCC: maximum clade credibility

MCMC: Markov chain Monte Carlo

MEME: mixed effects model of evolution

ML: Maximum Likelihood

MRCV: Michigan rabbit calicivirus

mRNA: messenger ribonucleic acid

mtDNA: mitochondrial deoxyribonucleic acid

mya: million years ago

MYXV: myxoma virus

NNI: nearest neighbor interchange

NoV: *Norovirus*

nt: nucleotides

NTP: nucleoside triphosphate

OD: optical density

ORF: open reading frame

OSGE: O-sialoglycoprotein endopeptidase

P: protrusion

PAA: polyacrylamide

PBS: phosphate buffered saline

PCR: polymerase chain reaction

PDB: protein data bank

PDV: phocine distemper virus

PGA: printed glycan array

PNGase F: peptide-*N*-glycosidase F

PS: parsimony score

R_0: basic reproduction number

RCV: rabbit calicivirus

RdRp: RNA-dependent RNA polymerase

REL: random effects likelihood

RHD: rabbit hemorrhagic disease

RHDV: rabbit hemorrhagic disease virus

RNA: ribonucleic acid

RT-PCR: reverse transcription polymerase chain reaction

S: shell

SARS CoV: severe acute respiratory syndrome-related coronavirus

sgRNA: subgenomic ribonucleic acid

SIV: simian immunodeficiency virus

SLAC: single likelihood ancestor counting

SNP: single nucleotide polymorphism

SPR: subtree pruning and regrafting

ssRNA: single-stranded ribonucleic acid

Ta: annealing temperature

TMB: 3,3′,5,5′-tetramethylbenzidine

tMRCA: time to the most recent common ancestor

TNF-α: tumor necrosis factor alpha

UTR: untranslated region

VLP: virus-like particle

VPg: viral genome-linked protein

Thesis framework

Two viral diseases, rabbit hemorrhagic disease (RHD) and European brown hare syndrome (EBHS), are the main causes of the wild populations' decline of European rabbit (*Oryctolagus cuniculus*) and European brown hare (*Lepus europaeus*), respectively. These are valuable ecological and economic species due to their role as prey and game species. The etiological agents of RHD and EBHS are rabbit hemorrhagic disease virus (RHDV) and European brown hare syndrome virus (EBHSV), respectively, and belong to the genus *Lagovirus*, family *Caliciviridae*.

Understanding the mechanisms at the origin of virulence emergence of lagoviruses is crucial to study the host resistance. The work presented in this dissertation was thus integrated in the project entitled "Evaluation of the virulence of RHDV (rabbit hemorrhagic disease virus) and mechanisms of host resistance" (FCT-ANR/BIA-BIC/0043/2012), supported by the Fundação para a Ciência e Tecnologia (Portugal) and the Agence Nationale de la Recherche (France).

The work developed in this thesis aimed at contributing to the current knowledge on the host-pathogen interactions of lagoviruses. Therefore, the evolution of the virus and the genetic aspects of viruses that might be relevant for host resistance are the main subjects presented here. The thesis is organized in five chapters and includes nine scientific manuscripts, of which seven were already published in journals indexed in the Science Citation Index (SCI) and two are in preparation.

The first chapter, entitled *Chapter 1. General Introduction*, contains a revision of the literature on key aspects of host-pathogen interaction: (i) co-evolution and the Red Queen hypothesis, (ii) virus attachment and entry to the host cells, (iii) recombination in RNA viruses and how it generates variability, and (iv) virus host shifts as a mechanism to expand host range. A brief revision of the main findings on the epidemiology of RHD and EBHS and on the evolution and genome organization of RHDV and EBHSV is also presented. Finally, the phylogeographic history of leporids is discussed, focusing on the historical events associated with leporids in Europe.

The recent emergence of a new RHDV variant in France that rapidly spread to other European countries was the main topic of our research on RHDV for this thesis. After being described in Spain and Italy, the emergence of the new RHDV variant in Portugal allowed us to study its dissemination patterns, characteristic features and associated epidemiology. A complete analysis of full-length genomes of Iberian strains revealed the existence of several events of recombination. The recombination pattern of the new RHDV variant allowed us to trace the introduction of the new RHDV variant to the Portuguese archipelago of Azores, and to determine that anthropogenic factors are likely at the origin of the arrival of the new RHDV variant to Azores. The resulting scientific manuscripts are compiled in *Chapter 2. Insights into the emergence and evolution of the new RHDV variant in Portugal* and are the following:

Paper I. Abrantes J, Lopes AM, Dalton KP, Melo P, Correia JJ, Ramada M, Alves PC, Parra F, Esteves PJ: **New variant of rabbit hemorrhagic disease virus, Portugal, 2012-2013**. *Emerging Infectious Diseases* 2013, **19**:1900-1902.

Paper II. Lopes AM, Correia J, Abrantes J, Melo P, Ramada M, Magalhães MJ, Alves PC, Esteves PJ: **Is the new variant RHDV replacing genogroup 1 in Portuguese wild rabbit populations?** *Viruses* 2015, **7**:27-36.

Paper III. Lopes AM, Dalton KP, Magalhães MJ, Parra F, Esteves PJ, Holmes EC, Abrantes J: **Full genomic analysis of new variant rabbit hemorrhagic disease virus revealed multiple recombination events**. *Journal of General Virology* 2015, **96**:1309-1319.

Paper IV. Almeida T, Lopes AM, Magalhães MJ, Neves F, Pinheiro A, Gonçalves D, Leitão M, Esteves PJ, Abrantes J: **Tracking the evolution of the G1/RHDVb recombinant strains introduced from the Iberian Peninsula to the Azores islands, Portugal**. *Infection, Genetics and Evolution* 2015, **34**:307-313

The *Chapter 3. EBHSV: virus evolution and attachment factors* starts with the description of a genome walking approach for obtaining a full-length genome sequence of the virus and allowed comparison with RHDV. Next, the molecular evolution of EBHSV was assessed by looking for signatures of positive selection, by estimating the time to the most recent common ancestor (tMRCA) and by performing an epitope mapping.

Finally, the study of EBHSV attachment to its host revealed interesting differences in the binding of these lagoviruses. The results are presented in three scientific papers as follows:

Paper V. Lopes AM, Gavier-Widén D, Le Gall-Reculé G, Esteves PJ, Abrantes J: **Complete coding sequences of European brown hare syndrome virus (EBHSV) strains isolated in 1982 in Sweden**. *Archives of Virology* 2013, **158**:2193-2196.

Paper VI. Lopes AM, Capucci L, Gavier-Widén D, Le Gall-Reculé G, Brocchi E, Barbieri I, Quéménér A, Le Pendu J, Geoghegan JL, Holmes EC, et al.: **Molecular evolution and antigenic variation of European brown hare syndrome virus (EBHSV)**. *Virology* 2014, **468-470**:104-112.

Paper VII. Lopes AM, Lora M, Le Moullac-Vaidye B, Ruvoën-Clouet N, Breiman A, Galanina OE, Bovin NV, Esteves PJ, Abrantes J, Le Pendu J: **A new attachment factor in the *Lagovirus* genus: the European brown hare syndrome virus (EBHSV) uses *N*-acetylglucosamine O-glycans to attach to host cells**. In preparation.

Virus evolution is often punctuated by host shift events, allowing the virus to establish and adapt to a naïve population. Spillover events were not known for RHDV, but a study conducted on Iberian hares with symptoms characteristic of a *Lagovirus* infection revealed an infection by an RHDV strain circulating in Portugal at the time of sample collection. Two competing hypotheses for the emergence of RHDV and EBHSV had been postulated: either the evolution from non-pathogenic forms or a species jump from *Sylvilagus floridanus*. The binding patterns and glycan affinities of pathogenic and non-pathogenic strains were analyzed in light of the two hypotheses. The results of these works are in *Chapter 4. The impact of host shifts in* Lagovirus *evolution* and are the following:

Paper VIII. Lopes AM, Marques S, Silva E, Magalhaes MJ, Pinheiro A, Alves PC, Le Pendu J, Esteves PJ, Thompson G, Abrantes J: **Detection of RHDV strains in the Iberian hare (*Lepus granatensis*): earliest evidence of rabbit lagovirus cross-species infection**. *Veterinary Research* 2014, **45**:94

Paper IX. Lopes AM, Lora M, Le Moullac-Vaidye B, Galanina OE, Breiman A, Strive T, Marchandeau S, Le Gall-Reculé G, Bovin NV, Ruvoën-Clouet N, Esteves PJ, Abrantes J, Le Pendu J: **Leporids tissue binding and glycan specificities of the new RHDV variant and non-pathogenic related viruses**. In preparation.

In *Chapter 5. Final considerations*, the results obtained within the thesis are analyzed. The major conclusions and insights of this dissertation, as well as the implications for future research, are presented, and future directions of the work are also discussed.

Chapter 1

General Introduction

1. **Host-pathogen interactions**
2. **Lagoviruses and associated diseases**
3. **The order Lagomorpha**
4. **References**

1. Host-pathogen interactions

Host-pathogen co-evolution

The dynamic interplay between the pathogen and its host is a powerful tool to study evolution and its practical implications in nature. The emergence of several RNA viruses, such as HIV, foot-and-mouth disease virus and barley yellow dwarf virus, represents major threats to animal and plant populations whilst providing examples of acting evolutionary forces [1-3], though the factors underlying their emergence are mostly still unclear. Whereas host extinction due to emerging pathogens is rare [4], endangered species may be at a higher risk [see for example the impact of Ebola virus on African apes; 5]. Both epidemiologists' and evolutionary biologists' research is focused on the mechanisms of RNA viruses evolution and on the epidemiological history of viruses. The complete understanding of the forces promoting virus origin, emergence, and spread, together with experimental analyses, may shed light on how viruses will evolve in the future.

Pathogens are part of natural systems and, as such, influence host genetic diversity and population dynamics. These effects on host fitness, together with their rapid spread and evolution, make pathogens key selective agents that are capable of either maintain host genetic variation or prompt genetic shifts in host traits [6]. Host evolution is linked to pathogen evolution and vice-versa, in a dynamic process of co-evolution (Fig. 1.1). Selection acts on the host as a result of the pressure to evolve resistance to pathogens. In turn, changes in pathogens occur in order to counteract host defenses, and are also driven by selection (Fig. 1.1). Traits involved in co-evolution are typically found at pathogen's loci related with virulence, resistance and host evasion, and at loci of the host related with the immune system and escaping to pathogens [7-9]. Woolhouse *et al.* [10] defined three pre-requisites for the occurrence of co-evolution: genetic variation in relevant traits, reciprocal effects on the fitnesses of the interacting species and dependence of the outcome of these interactions on the genotypes.

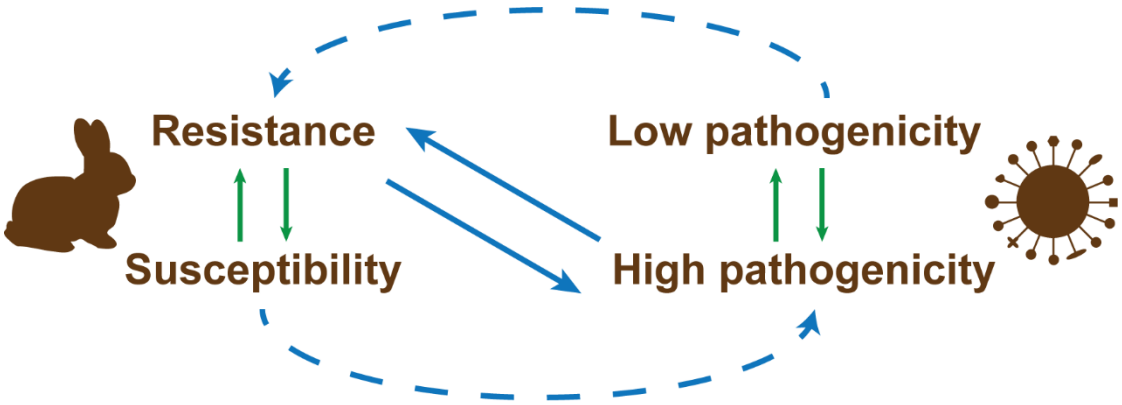

Figure 1.1 Schematic representation of co-evolution between host and pathogen. Green arrows indicate intra-specific selection; solid blue arrows indicate increased selection for host resistance/pathogen infectivity; dashed blue arrows indicate reduced selection for host resistance/pathogen infectivity. Adapted from [10].

In 1973, Van Valen proposed an evolutionary theory known as the Red Queen hypothesis, which owes its name to the character of the book *Through the Looking Glass*, by Lewis Carrol. This theory highlighted that the cyclical adaptation of hosts and pathogens maintains relative fitness constant ("It takes all the running you can do, to keep in the same place"), converting this in a 'zero-sum game' [11]. Recently, three classes of the Red Queen dynamics were defined (Fig. 1.2) [11], summarized as follows:

(i) *Fluctuating Red Queen*, whereby selection favors rare alleles. For instance, the pathogen is adapted to the most common host genotype and fails to infect hosts with a rare allele;

(ii) *Escalatory Red Queen*, whereby selection proceeds through selective sweeps, resulting in an escalation of traits. It is usually associated with polygenic or quantitative traits;

(iii) *Chase Red Queen*, whereby selection pressure reduces genetic diversity within populations but increases divergence between populations. Complex polygenic traits are associated with this mode of Red Queen dynamics.

The Red Queen hypothesis is challenged by multiple host-pathogen interactions that affect the selection pressures exerted on hosts and pathogens. For instance, the selection pressure acting on the pathogen may be diminished if it has multiple hosts and evolving infectivity in another host requires less effort than evading resistance of the

current host. This ultimately leads to host switching, which is favored by the ability of viruses to exploit, for example, different cell receptors [reviewed in 10].

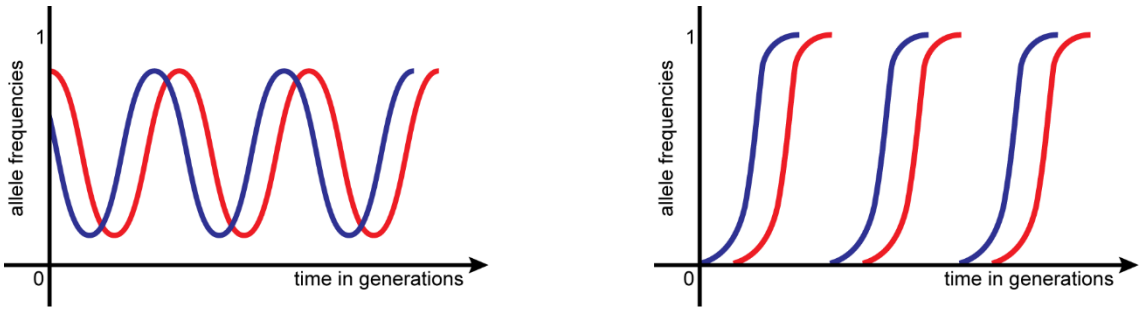

Figure 1.2 Allele frequencies associated with the Red Queen scenarios. On the left is shown the fluctuating selection mode of the *Fluctuating Red Queen*, associated with oscillations on the allele frequency dynamics. On the right, the selective sweeps of the *Escalatory Red Queen* and the *Chase Red Queen* are shown. Although presenting the same variation in the allele frequency dynamics, these two scenarios differ by the uni- or multi-dimensional character of the selection mode, respectively. The blue line represents the host alleles and the red line represents the pathogen alleles. Adapted from [11] and [12].

A trade-off between transmission and virulence also accompanies pathogen evolution [13]. Pathogens must evolve towards an intermediate level of virulence, which is favored because pathogens with low virulence do not produce sufficiently high viral loads and pathogens with high virulence kill hosts too fast, both situations compromising transmission. Although controversial, natural selection favors strains with the better balance between these two factors, which maximizes the basic reproduction number (average number of cases that a single virus strain generates on an uninfected population [14], measuring the transmission potential of a virus) of the pathogen [15]. However, the transmission mode influences virulence [16], as well as the period of intra-host evolution, i.e., if the host is infected by other strains the competition between strains will increase, and consequently virulence [17].

Infectious diseases have long been considered a major selective force shaping genetic diversity [18]. Nonetheless, demonstrating co-evolution in practice is challenging, since asymmetries in genetic variation and generation times, multiple hosts/multiple pathogens, phenotypic plasticity, environmental factors, etc, mask its effects [10]. The introduction of myxomatosis and rabbit hemorrhagic disease in Australia are valuable tools to study emergence and evolution of a pathogen in a naïve host population, by the study of host resistance and interaction with co-circulating viruses [19,20]. Moreover, the European rabbit is a model species for both nature and laboratory studies, facilitating the

identification of traits related to virulence (laboratory studies *per se* are sometimes limited since they do not mimic the conditions that occur in nature [21]).

Virus entry

Viruses drive their genome from infected to uninfected cells and their ability to spread determines the replication success. Virus life cycle is initiated with binding and entry to host cells, which can be hampered by several barriers. By overcoming the epithelial barriers of the respiratory, digestive or reproductive tracts (the most likely entry doors), viruses gain access to the host replication cellular machinery [22]. Interactions with receptors at the cell surface determine virus attachment, entry pathways and trafficking in the cell. Almost all mammalian cell types are targets for virus infection if possessing appropriate receptors. Their absence at the cell surface restricts infection, determines cell and tissue tropism and defines the nature of the virus-induced diseases [23].

The skin, mucosal surfaces and blood/lymphatic circulation are the first line of protection of the host against the virus, either through mechanical action or host factors, such as glycans or lectins [24]. Interestingly, whereas lectins play a role in the immune defense on one side, they can also be doors of entry and spreading for several viruses, as they are receptors for HIV-1, hepatitis C and Ebola viruses, among others [25-28]. A strategy of the virus to escape from the immune system is to protect its nucleic acid by a membrane and/or protein shell. In this case, viral particles are resistant to protect the genome but also unstable enough to ensure the release of the genetic cargo on the target cell [22].

The first interaction between the host and the pathogen is *via* attachment factors present in susceptible cells. Attachment factors might simply bind to the particles to establish a contact point at the cell membrane, helping to concentrate the virus on the cell surface [29]. Association between viral proteins and the cell is often electrostatic and thus lacks specificity, albeit some viruses have specific binding requirements [22]. To attach to host cell factors, the virus may form projections or depressions on its surface that will interact with the cell surface molecules.

The cell has on its surface a wide range of proteins, lipids and glycans that can be used for attachment and entry, including ion transporters, adhesion factors and signaling proteins (Fig. 1.3) [29]. Certain cell surface proteins, such as glycoproteins, are

attachment factors par excellence. These include proteins in the integrins' family and immunoglobulins, the latter mainly associated with cell adhesion [30]. Viruses that use the gastrointestinal tract commonly use glycolipids to bind to the target cells. Glycoproteins and glycolipids are present in the glycocalyx, which is frequently the first structure encountered by viruses at the cell surface [31]. The negatively charged nature of the glycocalyx is conferred by the presence of proteoglycans. The great diversity of proteoglycans is given by glycosaminoglycans (sulfated glycan side chains with variable degree of sulfation), which are attached to the core protein. One of the glycosaminoglycans widely used as a viral attachment factor is the heparan sulfate (Fig. 1.3) [e.g. 32,33-37]. Several authors identified viral consensus binding motifs to glycosaminoglycans that are mainly characterized by the presence of basic amino acids [38,39].

Cell surface carbohydrates also mediate cellular binding and are recognized by several viruses. For example, sialic acid is the first contact of Influenza virus with the cell [40], but other important pathogens also use sialic acid to attach to the host [41]. The long side chains of carbohydrates allow them to be relatively far from the cell surface, becoming likely to be primarily encountered by the virus. Furthermore, carbohydrates are expressed in a wide range of cell types and their charges attract viruses' charged residues promoting a rapid interaction. Similarly, glycans such as histo-blood group antigens (HBGAs) may function as virus attachment factors [42]. For example, human noroviruses interact in a first approach with HBGAs and with heparan sulfate [reviewed in 43].

When interactions with host attachment factors serve only as a foothold for the virus, specific receptors are then recruited. In the *Caliciviridae* family, for example, feline calicivirus (FCV; genus *Vesivirus*) attaches first to sialic acid and then uses a cell surface protein as a receptor, the junctional adhesion molecule 1 (JAM-1), for penetration and entry into the host cells [44-46]. Entry receptors will trigger signaling pathways in the cell, actively promote virus endocytosis and mediate conformational changes in the virus particle that lead to uncoating and release of the viral genome into the cell [30]. These interactions, unlike attachment, are highly specific, but of low affinity [47]. Receptors often accompany the virus into the cell, and penetration is achieved after a series of conformational changes triggered by exposure to low pH, lipid composition, proteolytic cleavage and redox reactions [reviewed in 23].

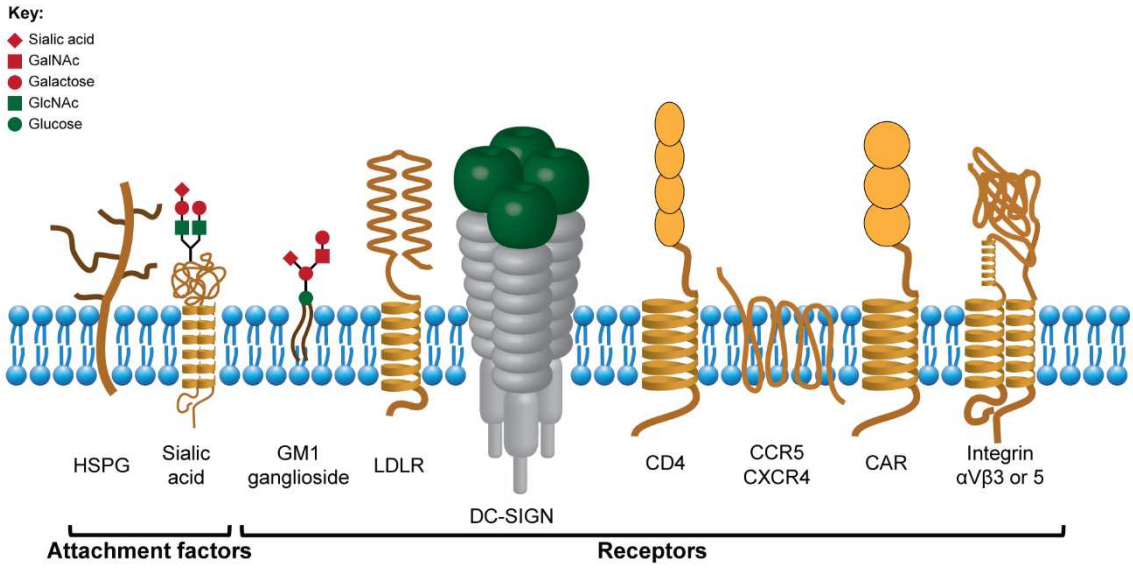

Figure 1.3 Main attachment factors and receptors used by viruses. HSPG: heparan sulfate proteoglycan; LDLR: low density lipoprotein receptor; DC-SIGN: dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin; CD4: cluster of differentiation 4; CCR5: C-C motif chemokine receptor type 5; CXCR4: C-X-C motif chemokine receptor type 4; CAR: coxsackievirus and adenovirus receptor. Adapted from [23].

Molecules used as entry receptors usually have critical roles in cellular function, thus are strongly conserved and selection acts on the virus to maintain binding to receptor motifs [31]. Nonetheless, viruses with high mutation rates are more apt to switch receptors or use alternative receptors in the absence of the primary receptor, as it was shown for Sindbis virus [48]. Viruses usually bind to multiple receptors, either at the same time or in cascade, enhancing avidity and activating endocytosis. Different viruses might recognize the same cellular receptors, and, conversely, viruses from the same family might use different receptors [see a review in 22].

The attachment and entry of a virus to its host is an optimal target for drug application and, although complex, their potential as targets for antiviral therapeutics makes it worth to try new approaches of studying the virus-host interactions. Antiviral therapy may be used in two ways, either by blocking virus attachment through the use of synthetic molecules that bind to receptors, or by using synthetic receptors that bind to the virus and block attachment. Nonetheless, these approaches have some limitations due to the ability of viruses to escape the blocking effect by generating resistant variants. Furthermore, the interactions between viruses and receptors are determinants in viral tropism and associated with host switching, an important aspect of virus-host interactions that will be discussed in detail further on.

Recombination as a source of variability in RNA viruses

The high mutation rates of RNA viruses, combined with their short generation times and large population sizes, make them highly variable. Along with mutation, recombination is an important mechanism in RNA viruses evolution as it quickly generates genetic variability [49]. Such importance is reflected in the fact that several emerging viruses are the result of past recombination events between genetically distinct viruses [50-52]. Briefly, recombination is the process by which one 'donor' sequence exchanges genetic information with an 'acceptor' sequence, resulting in a mosaic RNA from at least two 'parents'. Recombination does not create new mutations in the genome, but creates new genome combinations, on a single infectious cycle, by reshuffling the ones that are already present in the population [53]. If this process occurs on viruses possessing a segmented genome and the entire genomic segments are exchanged, is termed reassortment, a very common process in Influenza virus [e.g. 54,55].

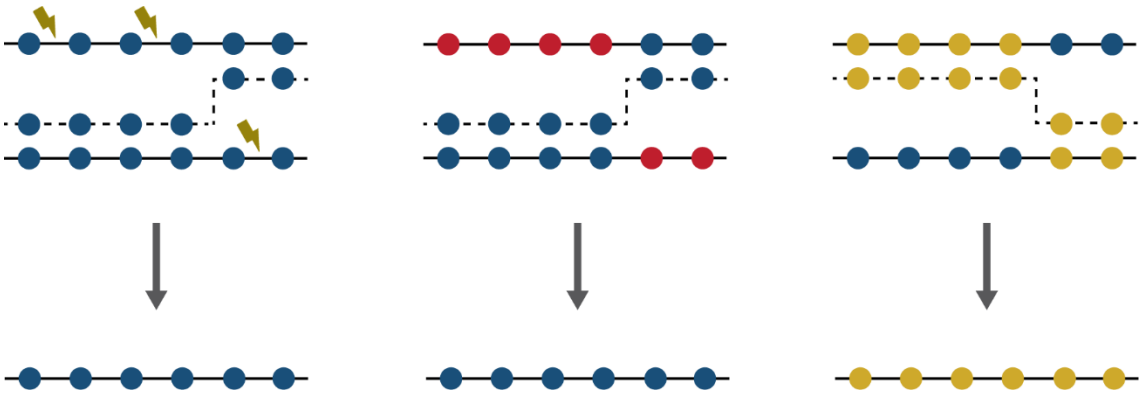

Figure 1.4 Evolutionary effects of recombination. On the left, recombination generated a functional genome from damaged parental strains; in the middle, recombination removed deleterious mutations (red circles); on the right, recombination created advantageous mutations (yellow circles). Blue circles indicate the wild type loci. Adapted from [56].

Higher recombination frequencies might quickly generate high fitness genotypes that are better adapted to evolving hosts, either by mixing viable genotypes or by eliminating deleterious mutations that can decrease virus fitness [57]. This generates adaptive value by acting as a genetic repair mechanism in viruses such as RNA viruses with a double-stranded genome. Depending on the parental genotypes, three scenarios may occur: (i) generation of a functional genome from damaged parental genomes; (ii) removal of deleterious mutations, leading to a selective advantage of the recombinant molecule; and (iii) generation of advantageous combinations of mutations, increasing the rate of adaptive evolution (Fig. 1.4) [56]. On contrary, RNA recombination might also be

disadvantageous if it affects the integrity of the viral genome. Recombination can create a high proportion of non-viable genomes due to simultaneously introduced deleterious mutations. As no clear answer is yet available, the hypothesis that recombination in RNA viruses is only a consequence of the replication machinery and mechanism or is driven by ecological circumstances cannot be excluded [58].

Considering the mechanism at the origin, recombination is divided in replicative (copy-choice) and non-replicative (breakage-rejoining) [59]. The replicative model involves the switch of the viral polymerase (RNA-dependent RNA polymerase or reverse transcriptase) from the 'donor' molecule to the 'acceptor' molecule during synthesis (Fig. 1.5). This is the most accepted model of RNA recombination and the most likely to produce viable molecules [59]. Template switching is influenced by sequence homology: higher degree of identity usually means higher probability of exchanging templates [60]. Secondary structures as hairpins also facilitate template switching. The viral polymerase stops at these regions, which thus constitute recombination hotspots. There are also terminators that release the viral polymerase from the donor template. The 'donor' template likely participates in template switching by forcing the pause of the viral polymerase and the 'nascent' RNA selects the reinitiation site on the 'acceptor' template [57].

In the non-replicative mechanism, RNAs are randomly cleaved by mechanical forces or by enzymatic activity, generating 3'-P and 5'-OH ends, and then self-ligated or ligated by cellular ligases (Fig. 1.5) [61]. This type of recombination is most likely to occur in double-stranded DNA viruses and, due to its stochastic nature, it is more likely to produce non-homologous recombinants [59]. Nonetheless, it has been experimentally confirmed for several RNA viruses such as flaviviruses, polioviruses and pestiviruses [62-64]. The breakage-rejoining mechanism may occur between fragments of a virus, different viruses and even between viral and cellular RNAs [61]. The non-replicative mechanism presumes recombination in the absence of a viral polymerase, since it occurs prior to the synthesis of the polymerase [63].

Based on the structure of the crossover junction, recombination is considered homologous or non-homologous [61]. If the breakpoint is on the same site on both parental molecules, recombination is homologous and the resultant molecule has the same structure as the parental strains. Strikingly, the crossover site might not be at the region with most sequence similarity [56]. The non-homologous recombination occurs between highly dissimilar genomic regions or molecules and often produces deleterious genotypes due to its aberrant structures, with insertions, deletions and/or duplications.

Homologous recombinants are the most likely to generate functional proteins and replicate in the cell, and consequently to produce viable progeny [61].

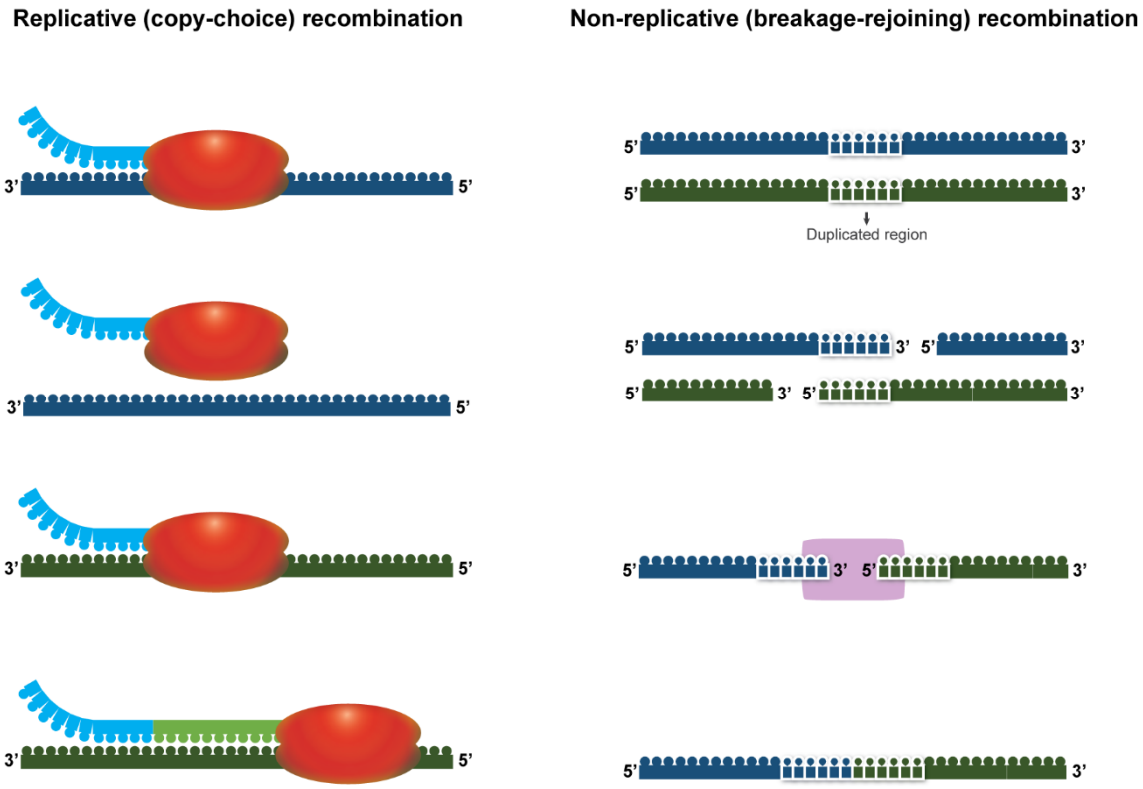

Figure 1.5 Mechanisms of recombination. On the left is represented the replicative (copy-choice) recombination, where the nascent strain is dissociated from the template and the polymerase finds another template to continue synthesis. On the right is represented the non-replicative (breakage-rejoining) recombination, where genomic fragments are cleaved and later rejoined, in this case by a cellular ligase. Adapted from [61].

Recombination is associated with emergence of new pathogenic circulating viruses and with increases in pathogenic potential and virulence of pre-existing strains [65-67], as well as to the expansion of viral host range [68,69]. Furthermore, it is also associated with alteration of transmission vector specificities and modification of tissue tropism, which ultimately lead to evasion of host immunity and evolution of resistance to antivirals [70,71]. Recombination is thus a mechanism associated with major changes during virus adaptation that has an impact on virus evolution, emergence and epidemiology.

There are several pre-requisites that determine the occurrence of recombination between strains. The first to be fulfilled is co-circulation of viruses in the same geographical area. In turn, this potentiates co-infection of the same individual/cell, as long as the prevalence of circulating strains is reasonably high [61]. Notably, viruses

associated with chronic infections might have higher rates of recombination due to the increased opportunity of co-infections [56]. In the cases that viral recombination is replication-dependent, high viral loads in the host organism will increase the probability of exchange genetic information [72]. The genetic homology between strains is also important, and closely related strains are more likely to produce viable recombinants [e.g. 73,74]. Finally, the genome structure and organization of the virus can also facilitate recombination. This is the case of several pseudodiploid viruses, for example, that package two viral genomic RNAs in a single virion, allowing the co-existence of different strains in the same virion; the recombinants are progeny of these heterozygous virions. If the recombinant represents an evolutionary advantage relatively to the parental genomes, it will become predominant in the population due to natural selection [61].

Frequencies of recombination are extremely variable between viruses [53]. Negative-sense, single-stranded RNA viruses are the less prone to recombine, probably due to their RNA-packaging mechanism. On contrary, positive-sense, single-stranded RNA viruses vary in the extent of recombination, with *Picornaviridae* showing high rates and *Leviviridae* with no reports of occurrence of recombination [56,75]. The highest recombination rates are found in retroviruses, such as HIV, which is an exceptional case, with recombination rates exceeding the mutation rates [76,77].

Recombination also has a role in species jumps. Cross-species transmission is relatively common in RNA viruses, and the adaptation to a novel host might be assisted by recombination, in the way that several genome combinations are tested [49]. Consequently, the likelihood of having a virus strain with increased fitness for the new host will increase. Remarkable examples of recombination associated with host shifting include Influenza A virus [55], SARS CoV [50] and turkey coronavirus [78]. However, establishing a direct link between recombination and host jumps is often difficult.

Recombination is undetectable when very similar strains recombine, but it can be detected when enough genetic variation exists. Evidences for recombination are found through the phylogenetic incongruences generated by the recombinants, albeit more powerful methods are available, involving the detection of substitution patterns or incompatibility among sites [79]. If recombination is not taken into account, the inferred phylogenies will not represent the true history of that particular region/gene (Fig. 1.6), depending on the relatedness of the sequences and on the size of the affected regions [80,81]. Older recombination events might be more obscure and difficult to trace, since subsequent mutation mitigates their effects [79]. Another problem arises with the advent of the new techniques of Next Generation Sequencing. This comprehensive analysis of

the genome relies on the assembly of very short fragments into contigs, which may produce artefactual recombination signals [82].

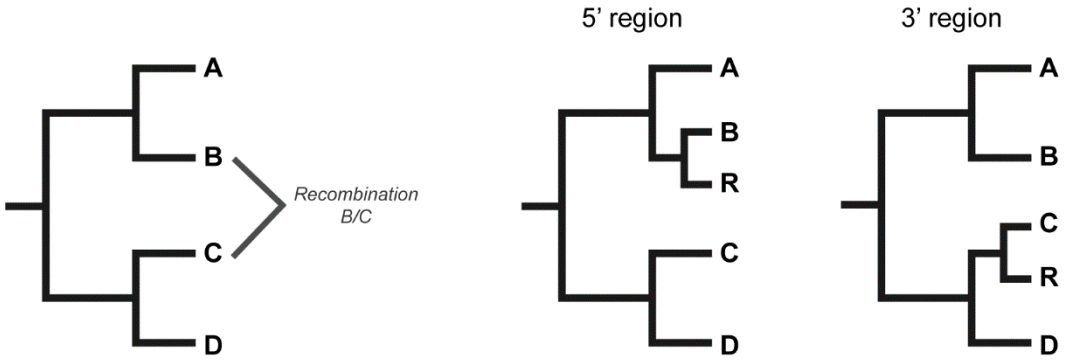

Figure 1.6 Effect of recombination on phylogenies. If recombination occurs between "B" and "C" (with "B" as donor template and "C" as acceptor template), it generates a recombinant "R" that clusters with "B" at the 5' region of the recombination breakpoint and with "C" at the 3' region. Adapted from [81].

In conclusion, there is growing evidence of the consequences of recombination in animal, plant and bacterial RNA viruses, by influencing evolution and epidemiology. Whether recombination represents a significant phenomenon in viruses' evolution is currently unknown, but recombination events are likely to be underestimated [79]. Understanding recombination events is critical due to their role on RNA virus emergence, but also for clinical practice, namely in evaluating the efficacy of drug therapies and vaccine campaigns.

Crossing the species boundaries

When a pathogen successfully jumps from its original host to a novel species, the outcome is unpredictable, the most severe being the emergence of a new viral disease that can affect humans and wildlife. Host jumps are mostly short-term spillover events affecting few individuals, with no onward transmission [83], but can occasionally take the form of local outbreaks, with transmission chains at a limited scale or, eventually, develop epidemic transmission of the disease in the new host, causing severe damage [24]. The HIV jump from chimpanzees to humans [84], the Influenza A virus jump from birds to humans [85] and the Ebola virus, with a recent outbreak with devastating consequences [86], are some extreme examples of the latter (Table 1.1).

Table 1.1 Examples of viruses that transferred between hosts to gain new host ranges.

Virus classification	Family	Virus	Original host	New host
(-)ssRNA	*Filoviridae*	Ebola virus	Unknown (bats?)	Chimpanzees; humans
(-)ssRNA	*Henipavirus*	Hendra virus	Fruit bats	Horses; humans
(-)ssRNA	*Henipavirus*	Nipah virus	Bats	Pigs; humans
(-)ssRNA	*Orthomyxoviridae*	Influenza virus	Water birds	Pigs; horses; humans
(-)ssRNA	*Paramyxoviridae*	CDV / PDV	Canids	Seals
(-)ssRNA	*Paramyxoviridae*	Measles virus	Possibly cattle	Humans
(-)ssRNA	*Paramyxoviridae*	Rinderpest	Eurasian cattle	African ruminants
(-)ssRNA	*Rhabdoviridae*	Australian bat lyssavirus	Bats	Humans
(+)ssRNA	*Coronaviridae*	SARS coronavirus	Bats	Palm civets or related carnivores; humans
(+)ssRNA	*Flaviviridae*	Dengue virus	Old World primates	Humans
ssRNA-RT	*Retroviridae*	SIV / HIV-1	Old World primates, chimpanzees	Humans
ssRNA-RT	*Retroviridae*	SIV / HIV-2	Sooty mangabey	Humans
ssDNA	*Parvoviridae*	FPV / CPV	Cats or similar carnivores	Dogs
dsDNA	*Leporipoxvirus*	Myxoma virus	Brush rabbit; Brazilian rabbit	European rabbits
dsDNA	*Poxviridae*	Monkeypox virus	Prairie dogs	Humans
dsDNA	*Poxviridae*	Smallpox virus	Other primates or camels (?)	Humans

CDV: canine distemper virus

PDV: phocine distemper virus

SIV: simian immunodeficiency virus

HIV: human immunodeficiency virus

FPV: feline panleukopenia virus

CPV: canine parvovirus

For the virus to achieve successful infection and transmission in the novel host and become established, three types of interactions are mandatory: contact points between hosts, virus-host interactions and interactions within the new host [87]. The first contact between the donor/infected host and the recipient host is affected by factors such as host distribution, host ecology and behavioral separation of the species involved in the cross-transmission (Fig. 1.7). Several of these factors have an anthropogenic cause, such as species movements/introductions and agricultural development, although natural causes can be at the origin of new contacts between hosts, e.g., climate changes [88,89]. Furthermore, a role for intermediate and amplifier hosts is also predicted for SARS CoV and Influenza A virus, for example [90,91]. All these changes bring together species that were not formerly in contact.

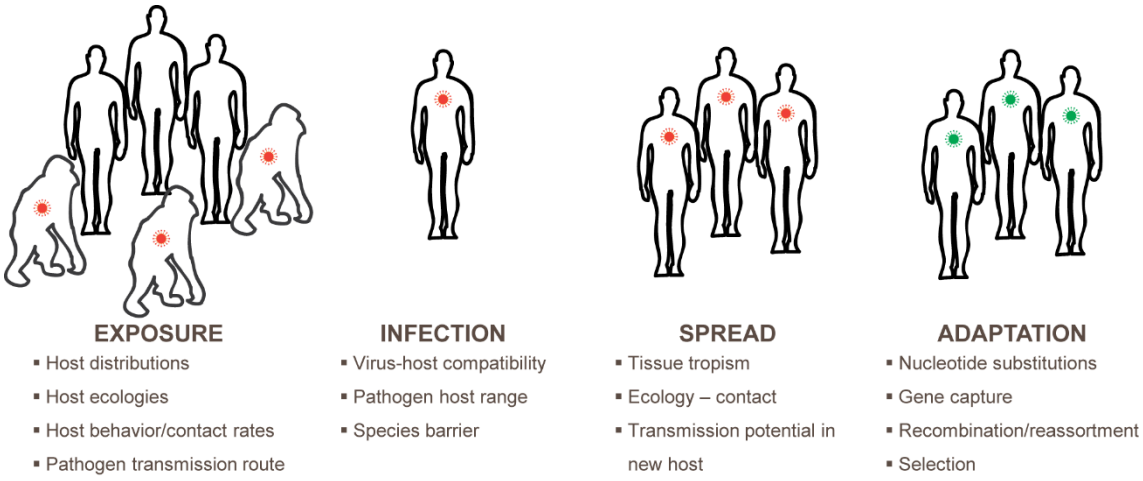

Figure 1.7 Types of interactions and steps involved in a virus host jump. For each step, the main host and pathogen factors/processes involved in the host switch are listed. Based on data from [87] and adapted from [24].

The mode of transmission of a “jumping” virus might be important in its ability to successfully switch host. While sexually transmitted or blood-borne viruses have lower probability of exposure, respiratory or vector-borne viruses are more likely to be successful [49]. However, the higher probability of host exposure to vector-borne viruses does not necessarily correlate with the ability of these viruses to establish infection. Their stronger selective constraints and antagonistic pleiotropy (beneficial mutations for one organism are detrimental to another) may limit their ability to host jump [12,92,93].

Considering the new host population, if the basic reproduction number, R_0, is greater than 1, each initial infection generates at least two secondary infections and there is potential for an epidemic; if R_0 is lower than 1, the initial case is not capable to replace itself and (even when the pathogen frequently jumps to the new host population) only minor outbreaks occur [94]. R_0 changes in function of host ecology, behavior, environment and phenotype [87]. The pathogen host range is also important when studying the possibility of host jumps. While specialist viruses (with one or a few natural hosts) are restricted by the receptors and replication mechanisms of a new host, generalist viruses (with several natural hosts) are adapted to different host cell mechanisms, thus it is expected an increased likelihood to host jump [24].

The closer the natural and the potential new host species are, the more likely is to occur a successful species jump (Fig. 1.8) [95,96]. Although susceptibility of potential hosts can vary, the phylogenetic proximity of hosts brings a more similar environment for the pathogen regarding cell types, receptors and other key components [96]. Besides phylogenetic relationships, which are undoubtedly a force guiding the success or failure of host switches, the intensity of contact between hosts and the density of the new host to promote onward transmission are also of extreme importance [24].

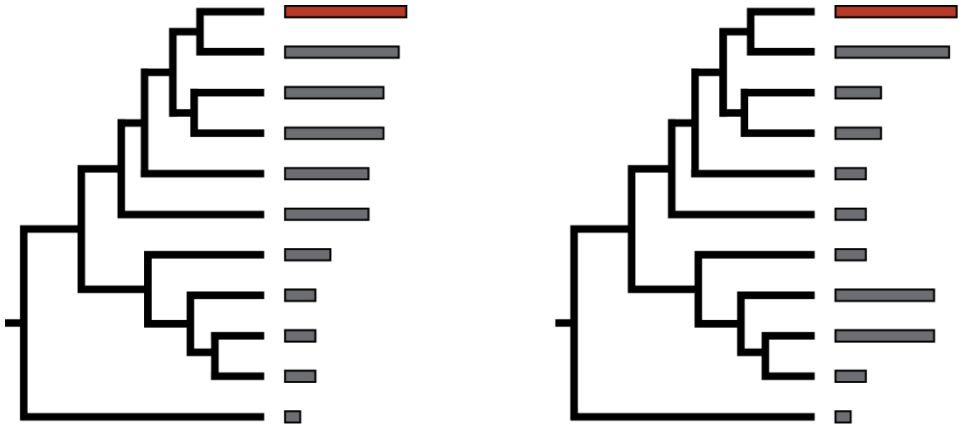

Figure 1.8 Effect of host relatedness in the pathogen's ability to jump between hosts. The length of the bars at the tips of the trees correlates with the success of pathogen infection; bars in red correspond to the natural host. In the tree on the left, the success of the pathogen is successively lower with higher host phylogenetic distance. In the tree on the right, highly susceptible clades are scattered across the host phylogeny, independently of the phylogenetic distance from the natural host. Adapted from [97].

The rule of closer phylogenetic hosts/higher probability of jumping between hosts is often challenged, since closely related species may have cross-immunity that protects

the new host (Fig. 1.8) [24]. Moreover, the geographic proximity and probability of exposure are not directly correlated with the phylogenetic relatedness. For example, humans are phylogenetically closer to other primates, but have greater exposure to rodents and, thus, are more likely to become infected by a pathogen that has in rodents its natural reservoir [98]. Indeed, the contact between viruses and a new potential host is occasionally prevented by the limited contact between the hosts [24]. There are several examples of viruses that successfully infected species that are phylogenetically distant from their natural hosts, including the SARS CoV-like virus from bats to humans [99] and the tobacco ringspot virus from plants to honeybees [100].

Changes in host receptor binding are key in the virus adaptation process, so that the virus might lose or reduce its ability to bind to the natural host [97]. Indeed, cell and tissue tropism is the result of the interactions between the virus and host cell receptors (Fig. 1.7) [101]. Those first interactions determine host specificity and several changes in the virus might occur until it is adapted to the new host, even if it does not ensure sustained transmission [58]. For example, influenza A virus has in birds its natural host and main reservoir. Usually, it is not capable to infect humans but, when it does, human-to-human transmission is not possible and 13 mutations would be required [102]. On contrary, a minor genetic change of a single mutation is enough for the Venezuelan equine encephalitis virus to jump from rodents to horses [103]. Similarly, in order to bind to canine receptors, the capsid gene of feline parvovirus gained at least two mutations which prevented its ability to infect cats, although it was later recovered [104,105].

After attachment to the host cell surface receptors, other barriers need to be overcome by the virus, i.e. entry in the cell, trafficking and replication using the host cell's machinery, where it may encounter several intracellular restriction factors. Finally, virus shedding is crucial for propagation to other cells of the new host. The virus also needs to avoid or suppress the host immune antiviral response. When viruses start infection after a host jump, they are usually not well adapted, and hence replicate less and transmission is not as efficient [24]. The level of infectivity, length of infection and transmission period, and level of genetic variation also play a role in adaptation to the new host. The time spanning until the entire process of adaptation to a new host is complete varies, with some viruses taking years until they are fully adapted and others, which do not require evolutionary changes on the virus, being more time-efficient.

Pathogen emergence in the new host species is difficult to predict and hence effective surveillance is the most efficient way to monitor it. However, there are some predictions that can be made based on past events, which show that the majority of

pathogens able to cross the species boundaries are single-stranded RNA viruses [e.g. 84,86,106]. RNA viruses are exceptional candidates to switch hosts due to their high mutation rates, short generation times and large population sizes [58,87]. Thus, their rapidly evolving genomes are capable of quickly adapting to a new host and explain why they are prone to host shift. While most mutations can be deleterious, other beneficial mutations will be selected to optimize the use of cellular machinery, virulence, transmission potential and also to avoid immune system responses [97]. Adaptation to a new host is dependent on a multitude of factors and, in many cases, these viruses only establish spillover infections and do not completely adapt to the new host [107].

Phylogenies recovered from several pathogens evidence that host shifts are relatively frequent in their evolution [97]. Indeed, if the phylogeny of the virus does not mirror the phylogeny of the natural host, it is therefore likely that a host shift occurred in the past. Those phylogenetic incongruences are very common and long-term co-speciation is a rare phenomenon [108]. Predicting where, when and what pathogens will emerge in a near future and whether a host jump will be a spillover or will largely spread is of extreme importance to prevent emerging diseases and epidemics, but also a difficult task and prone to fail, leading to an inappropriate use of resources [98]. This understanding of how ecological disturbance affects genetics, ultimately leading to a host shift, will give clues on the origin of new viruses.

2. Lagoviruses and associated diseases

Rabbit hemorrhagic disease (RHD)

The Jiangsu southern province of People's Republic of China was the first to acknowledge a new viral disease in European rabbits (*Oryctolagus cuniculus*, Linnaeus, 1758) in 1984, with probable origin in Angora rabbits imported from Germany [109]. Then, the disease rapidly spread to the entire country, as well as to Korea, due to rabbit fur importation from China. Two years later, rabbit hemorrhagic disease (RHD) reached Europe through Italy [110]. From there, RHD spread to several other European countries, affecting wild and domestic rabbits [reviewed by 111,112]. In the Iberian Peninsula, the disease was first reported in 1988 in Spain [113] and in 1989 in Portugal [114]. In 1988, contaminated frozen rabbit carcasses imported into Mexico brought RHD to the American continent. Currently, RHD has been reported in all continents and is endemic in most parts of the world [115].

RHD is a highly contagious, acute and fulminating disease, being in most cases fatal in adult rabbits. About 30 vertebrate species were used to test host range, but all of them failed to produce disease [116]. Thus, only *Oryctolagus cuniculus* seems to be susceptible to RHD, albeit other species may seroconvert or act as reservoirs [117,118]. The incubation period of RHD varies between one and three days and the clinical evolution of the disease can be classified into peracute, acute, subacute or chronic.

In the RHD peracute form, no clinical signs are observed [119]. On contrary, rabbits with an acute infection present several clinical signs and usually die 12-36h after the onset of fever. Clinical signs include some neurological signs (such as convulsion, ataxia and paralysis), dullness, prostration, apathy, anorexia and, 24h before death, they can start to present increased heart and respiratory rates. Bloody diarrhea, blood in the nares and/or mouth and hematuria may also be present [115,119]. In the subacute course, rabbits present the same but milder symptoms, surviving for a week or more and in some cases recovering. When an outbreak occurs, 5-10 % of the rabbits may show a chronic evolution of RHD, characterized by severe jaundice, loss of weight and lethargy. Rabbits may either die one or two weeks later, likely because of liver dysfunction, or recover and seroconvert, developing high titers of antibodies against the virus that last for at least one year [120]. Rabbits that survive may present chronic liver disease, e.g., cirrhosis.

Circulatory and degenerative disorders are the main pathological lesions associated with RHD, including liver necrosis and splenomegaly. The liver appears pale, swollen and with a marked lobular pattern; periportal hepatocytes are necrotic, and damage may extend further to midzonal or internal areas. The spleen is swollen as well, engorged with blood and with rounded edges. In general, viscera often present fibrin thrombi, and are congested and hemorrhagic due to massive disseminated intravascular coagulation (DIC). The tracheal mucosa is hyperemic, containing abundant bloody foam. Discoloration of the ears, conjunctiva and subcutis are visible in the subacute and chronic courses of RHD [112,121,122].

Severe leukopenia (decrease in lymphocytes and heterophils) and marked increase of liver enzymes (lactate dehydrogenase, alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase and γ-glutamyltransferase) and proinflammatory cytokines (TNF-α) are observed a few hours prior to death. Serum bilirubin and triglyceride concentration are also abnormally high [123-126]. Histologically, lymphocytes' depletion and apoptotic cell death of hepatocytes is visible at the periphery of the lobule. Liver cirrhosis is also observed in rabbits with a subacute or subclinical form of RHD [127].

Due to a great capacity of spread, RHD causes severe losses in farms, with a strong impact on the economy that relies on rabbit fur and meat production, particularly in Asia and central Europe [128]. The ecological impact of RHD is also of concern, since the decrease in wild rabbit populations impacts the survival of rabbit-dependent predators, some of them highly endangered [129,130]. Mortality rates vary between 70 and 90 %, but can reach up to 100 % in adult rabbits from naïve populations. Susceptibility to RHD varies according to the age of the animals. Animals younger than two months of age do not develop RHD, probably because they acquire maternal passive immunity, but support some viral replication [131,132]. Susceptibility begins in the 5-6th week of life, with only 50 % of these rabbits producing infection and disease, and progressively rabbits become more susceptible up to the 8-9th week when they are fully susceptible [112].

Differences in the innate immune response triggered after RHD infection may account for the distinct resistance/susceptibility to infection of young and adult rabbits [123,133]. Indeed, young rabbits develop an adequate inflammatory immune response that may limit viral replication and allow the rabbit to establish a specific immune response against the virus [134], while adult rabbits die too quickly and do not have time to mount the specific response. Strikingly, when immunosuppressed young rabbits are

challenged, the resistance is lost and fulminant hepatitis causes death after three days [135], further confirming the importance of the innate immune response in the resistance against the virus.

The impact of RHD is dependent on several factors. Interaction with myxomatosis, rabbit population dynamics and the time of the breeding season are related with RHD incidence [136,137]. Arid regions are more affected than areas with higher rainfall in Australia, most probably due to cross-protection induced by circulating non-pathogenic rabbit caliciviruses [138,139]. In Australia, rabbits are an ecological and economic pest and disease introduction was equated as a biological agent to control rabbit numbers. In 1995, the virus escaped from quarantine, reached the mainland and rapidly spread [140]; in some areas, rabbit numbers fell by ~ 95 % [130].

On contrary, in southern Europe, rabbit conservation is of major interest, not only because rabbit is a keystone species in the Mediterranean ecosystem, but also due to its importance as a game species, and dramatic decreases in rabbit numbers represent a serious concern [129,141]. In the old continent, translocation of rabbits should be prevented, and meat and fur importation from RHD endemic areas should be highly restricted. While in wild populations eradication is not possible, in domestic rabbits quarantine, surveillance, vaccination and proper sanitary measures may eradicate the virus. Interestingly, Mexico managed to eradicate the disease with an intense control program [142]. The recovery of the populations after an outbreak is also multifactorial, with hunting pressure, initial rabbit densities, food availability and predator presence influencing the outcome [143-145].

A rational vaccination program is the most efficient way to control outbreaks in rabbitries, since no available treatment exists. However, experimental treatments were able to reduce liver damage and increase survival rates [146,147]. Commercial vaccines are available since the 1990s and provide protection for up to 12 months; booster vaccination guarantees further protection. After vaccination, rabbits produce systemic but not mucosal immunity, therefore, no protection against infection is ensured. Vaccines are produced with inactivated virus from liver suspensions of infected rabbits; alternatively, there are recombinant myxoma virus vaccines expressing the RHD virus capsid protein. The efficacy of vaccination programs was assessed in wild populations, but the results were quite discouraging [148].

Rabbit hemorrhagic disease virus (RHDV)

The etiological agent of RHD is rabbit hemorrhagic disease virus (RHDV), a *Lagovirus* belonging to the family *Caliciviridae*. This family includes also the genera *Nebovirus*, *Sapovirus*, *Vesivirus* and *Norovirus* [149]. RHDV virions are spherical and non-enveloped particles, whose diameter varies between 27 and 35 nm. Particles are arranged in a T = 3 icosahedral symmetry and present the characteristic cup-shaped depressions of the caliciviruses (Fig. 1.9b and c) [150,151].

The capsid protein VP60, a ~ 60 kDa molecule, is composed of an N-terminal arm, a shell (S) and a protrusion (P) domains. The S domain protects the inner RNA, and the P domain, further subdivided into P1 and P2, is exposed and is mainly associated with host cell attachment and antigenicity [152]. While the S domain is highly conserved and shares great sequence homology with the S domain of other caliciviruses, the exposed P domain is more variable among caliciviruses, due to host immune pressure, and the existence of putative pockets that bind to host cell receptors [153]. Consistent with this, signatures of positive selection were found in the P2 subdomain of the capsid protein [154].

RHDV is a polyadenylated, single-stranded and positive-sense RNA virus, with two open reading frames (ORFs) and a full genome of 7437 nucleotides (Fig. 1.9a) [155]. ORF1 (nucleotides 10-7044) codes for a polyprotein of ~ 257 kDa and 2344 amino acids that after proteolytic cleavage produces the non-structural proteins (including an helicase, a protease and the polymerase) and the capsid protein. ORF2 (nucleotides 7025-7378) is located at the extreme 3’ end, slightly overlapping ORF1, and encodes a minor structural protein, VP10, with 117 amino acids and ~ 10 kDa, which is a component of RHDV virions [156]. A subgenomic RNA of 2.2 kb at the 3’ end of the genome is the major source of VP60 production (Fig. 1.9a) [157]. Both genomic and subgenomic RNAs have a viral genome-linked protein (VPg) covalently attached to the 5’ end. The VPg and the 5’ untranslated region (UTR) might play a role in RHDV replication and/or translation [158].

The oral, nasal or conjunctival are the most likely routes of RHDV transmission. Experimentally, subcutaneous, intramuscular and intravenous routes are also possible [159]. Infected rabbits shed RHDV in all secretions and infected carcasses, contaminated food, bedding and water are also sources of the virus [111]. Indirect infection also occurs, for example through predators, which excrete RHDV derived from ingested infected animals, and scavenger birds that are able to spread the virus over long distances. Mechanical transmission occurs via fleas, flies and mosquitoes [160]. Notably, flies

feeding on rabbit carcasses were probably the vector responsible for facilitating RHDV escape from quarantine in Australia [160]. Finally, fomites, contaminated people and rabbit products or tissues help disseminating the virus. Virus transmission is facilitated by its high stability and resistance [161]. Indeed, RHDV remains infectious after several months in decomposing carcasses and clothes and is resistant to a wide range of temperatures and pH.

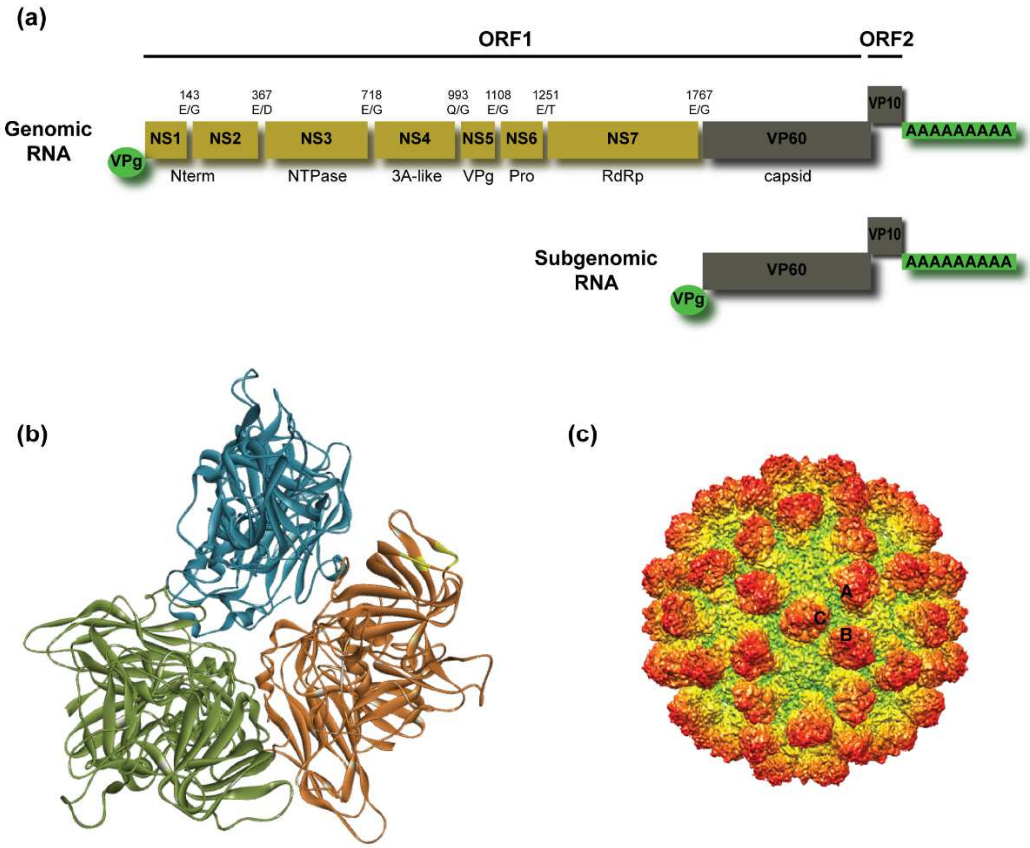

Figure 1.9 RHDV genomic organization and 3D arrangement of the capsid monomers. (a) Scheme of the organization of RHDV genome, with the expected cleavage sites and the encoded proteins indicated. ORF1 and ORF2 slightly overlap. A subgenomic RNA also produces the VP60 capsid protein and the minor structural protein VP10. **(b)** Model of the arrangement of the three capsomers that form the RHDV capsid. **(c)** Reconstructed cryoEM map of the RHDV virion. The letters A, B and C correspond to the arrangement of the capsomers shown in (b). (a) adapted from [162]; (b) and (c) adapted from [153].

In order to start the infectious process, RHDV uses HBGAs to attach to host cells. RHDV binds to A, B and H type 2 epitopes present on the epithelial cells of the upper respiratory and digestive tracts, although other receptors must be recruited to reach the main site of replication (hepatocytes), which do not express HBGAs on their surface [163,164]. At the moment no receptors are identified for RHDV. However, a few surface

protein genes that might facilitate virus entry into liver cells were identified [165], but remain to be tested. The affinity to different HBGAs is dependent on the RHDV strain and rabbits lacking these ligands are resistant to infection with low doses of RHDV [163]. Other caliciviruses, including human norovirus, also use HBGAs as attachment factors [e.g. 166,167]. Interestingly, HBGAs polymorphisms determine the ability of *Norovirus* strains to start infection [168].

Polymorphisms in the rabbit genes responsible for the synthesis of HBGAs, namely the α1,2-fucosyltransferase genes *Fut1*, *Fut2* and *Sec1*, and a possible association with resistance/susceptibility to RHDV, were investigated. An association between a weakly functional Sec1 variant and survival to an RHDV outbreak was observed, although it was always associated with functional Fut2 variants [169]. Additionally, an inactive Sec1 protein was shown to act as dominant-negative of the functional Fut1 protein that is the main contributor for the synthesis of RHDV attachment factors [170]. Ruvoën-Clouet and co-workers further hypothesized that young rabbits' resistance was associated with the HBGAs expression. Indeed, tissues of young rabbits lacked HBGAs expression and very weak binding was observed [164]. However, while HBGAs density is important to susceptibility to RHDV, their low expression confers only partial protection.

The lack of a cell culture system that efficiently replicates RHDV *in vitro* has been hampering epidemiological and virus transmission assays. Electron microscopy, enzyme-linked immunosorbent assays (ELISA), RT-PCR, hemagglutination and hemagglutination inhibition tests are some of the most widely used methods to determine RHDV infection. However, while it was firstly accepted that RHDV agglutinates human type O erythrocytes, in the last years some non-hemagglutinating strains were identified [171,172]. Moreover, this test can produce false-negative results in rabbits with a chronic form of the disease due to interference of anti-RHDV IgM and virus-like particles (VLPs) [159]. Determination of IgM, IgA and IgG titers by ELISA helps to distinguish between vaccinated, re-infected or first-infected rabbits [173]. Therefore, with this test, the population status and ability to resist infection can be assessed.

Throughout the years, several authors tried to assess the phylogenetic history of RHDV. However, its emergence is still not well understood and inference may be misguided due to recombination, a mechanism that has been described in RHDV strains from Europe and Australia [174-176]. Most phylogenetic studies relied on partial VP60 sequences although only full-length VP60 sequences provide enough information to a reliable and robust phylogenetic inference [177]. Several genogroups, following a temporal distribution, were identified in European strains, and a successive replacement

of older genogroups was observed in France [178,179]. These genogroups were nominated from G1 to G6 and form a single serotype with two subtypes: G1-G5 and G6 (Fig. 1.10), although G3, G4 and G5 genogroups were more recently considered a single group [177,180].

The genogroup G6 corresponds to RHDVa, an antigenic variant described in late 1990s in Italy and Germany [181,182], but already present in China since at least 1985 [180]. Cross-protection between RHDV and RHDVa is almost complete [181]. RHDVa is mainly associated with rabbitries and, in some areas, it seems to have replaced the original strains [183-185]. While in France the older genogroups were replaced, in the Iberian Peninsula only G1 strains are found in wild rabbit populations, although also evolving following a temporal pattern [186-188]. In addition, in the Portuguese archipelago of Azores a G3-G5 *like* variant was recently described, suggesting an independent evolution in the islands for more than 17 years [189,190].

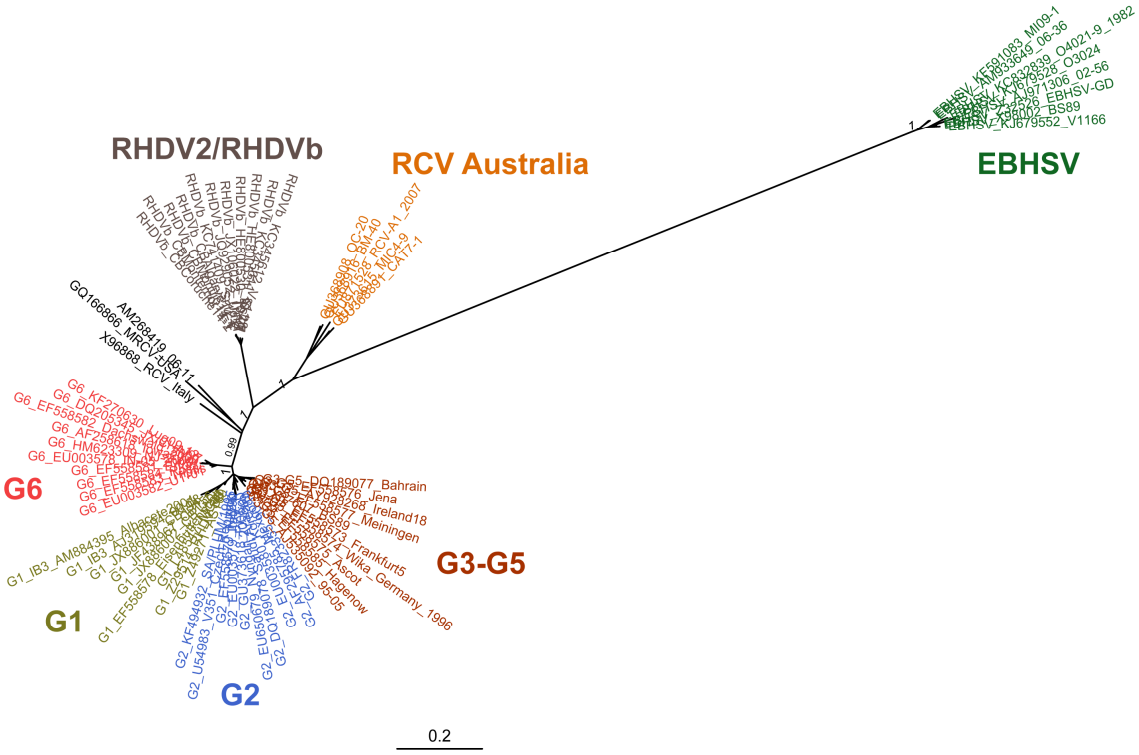

Figure 1.10 Phylogenetic relationships among lagoviruses. The tree was obtained using nucleotide sequences of the capsid gene from lagoviruses' strains representative of their diversity. The Maximum Likelihood method with 500 bootstraps and the GTR+G+I model were used. Only bootstrap values greater than 0.9 are shown.

After the initial description of pathogenic RHDV, non-pathogenic and moderately pathogenic lagoviruses started to be described. These strains are genetically related but distant from RHDV presenting > 20 % of nucleotide diversity (Fig. 1.10). The first evidences of the circulation of non-pathogenic viruses were reported in Europe, with serological studies showing that rabbit populations, where no mortality due to RHD was reported, presented antibodies against RHDV [191-193]. Non-pathogenic strains were later genetically characterized in Europe [194-197] and Australia [198]. An evolutionary analysis including the non-pathogenic Australian strain suggested that this non-pathogenic virus was present in the island for ~ 150 years, when the first wild rabbits arrived [199]. A moderately pathogenic lagovirus was identified in the USA [200], although its phylogenetic position and pathogenicity remain doubtful [200,201]. Non-pathogenic strains appear to provide a gradient of cross-protection against RHDV, from non-protective to fully protective [139,194,195,198,202], but cross-protection seems to depend on the time spanning between non-pathogenic and pathogenic infection and not on the titers of the antibodies that cross-react [139].

In the last few years, several attempts were made to estimate the RHDV substitution rate. Estimates range from 5.48×10^{-4} to 4.0×10^{-3} nucleotide substitutions per site per year (subs/site/year) [176,177,188,203]. Likewise, there is not a consensus on the time to the most recent common ancestor (tMRCA) estimates, which range from several decades to only a few years before RHDV emergence [177,180]. A very recent study from Eden and co-workers [204] reanalyzed RHDV sequences and showed that RHDV emerged in the late 1970s/beginning of 1980s, only shortly before the initial description in China. Interestingly, this emergence date is coincident with the introduction of the eastern cottontail (*Sylvilagus floridanus*, Allen, 1890) in Europe (see discussion below).

The factors that determine RHDV virulence are not known, but an association between mutations in the non-structural protein p16 and highly virulent strains was recently established [205], although the biological role of p16 remains to be determined. Other mutations might also be responsible for RHDV virulence, including at receptor binding, virus entry and virus replication [205]. Di Giallonardo and Holmes [21] pointed out that while MYXV, whose transmission is dependent on mosquitoes and fleas and live animals, has a trade-off between virulence and transmission, RHDV transmission relies both on the oral-fecal route and on flies feeding on animal carcasses, i.e., host death ensures transmission and hence requires a higher virulence. Similarly, non-pathogenic rabbit caliciviruses evolve towards low virulence, since the oral-fecal route does not require host mortality for transmission (Fig. 1.11) [21].

Because of its high virulence, RHDV provides a unique model to study the mechanisms of co-evolution between host and pathogen, such as the evolutionary changes occurring in the host to overcome the virulence of RHDV, and the variations in the virus that accompany host changes. The role of non-pathogenic circulating strains and of young rabbits in disease dissemination is starting to be considered [206,207] and remains a key to develop strategies to either increase rabbit numbers, in Europe, or control them, in Australia.

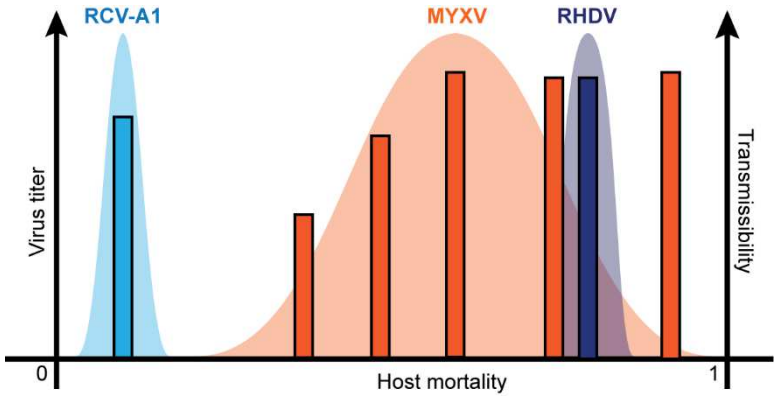

Figure 1.11 Possible relationship between virulence and transmission in rabbit viruses. Host mortality rates are a measure of virulence and range from 0 (benign) to 1 (always lethal). The bell-shaped distributions represent the likely relationship between mortality and hypothetical viral titers, represented by vertical bars. MYXV: myxoma virus; RHDV: rabbit hemorrhagic disease virus; RCV-A1: Australian rabbit calicivirus. Adapted from [21].

The emergence of a new RHDV variant

In 2010, unexpected outbreaks in rabbitries from north-western France caused high mortalities in vaccinated and non-vaccinated animals [208]. Additionally, wild rabbits from the same geographical region experienced severe outbreaks, with mortalities estimated in 80 to 90 %, numbers that resembled early RHDV mortalities in naïve populations in France [208]. Rapidly, the same unusual outbreaks were reported in Spain, Italy and Great Britain [209-211]. Sequencing of the capsid protein gene of the virus responsible for these outbreaks revealed ~ 80-82 % of nucleotide identity with other rabbit lagoviruses, including pathogenic (genogroups G1-G6) and non-pathogenic strains [211]. Thus, a new variant of RHDV was identified, that was related but highly distinct from the other known RHDV strains.

The epidemiology of RHD changed with the appearance of the new RHDV variant. Experimental results showed a longer course of the disease, highly variable but lower

mortality rates and higher occurrence of subacute/chronic forms [211]. Strikingly, the new variant infects and causes death in rabbit kittens, which present macroscopic lesions usually observed in adult rabbits [209]. Furthermore, it expanded the host range of RHDV by causing fatal hepatitis in cape hares (*Lepus capensis*, Linnaeus, 1758) and Corsican hares (*Lepus corsicanus*, de Winton, 1898), species where the older RHDV strains were never detected [212,213].

The efficacy of former commercial vaccines is limited against the new RHDV variant [208,211,214]. The limited immune response in vaccinated rabbits might be related with mutations at critical amino acids of the virus capsid protein. Indeed, a survey with several monoclonal antibodies showed different recognition patterns relatively to the older strains [211,212]. Analysis of the amino acid sequences of former RHDV strains and the new variant further showed that the majority of the amino acid differences reside in the C-terminus of the VP60, a region that constitutes the P domain and where the main antigenic regions are located [153,215]. Moreover, different epitopes are recognized by older RHDV strains and the new variant, and signatures of positive selection at sites previously detected for RHDV were not found for the new variant strains [154,177], although a new site was detected [214]. This site was located in the E region, according to the nomenclature of Neill [216], where the major antigenic determinants are thought to be located. More studies are required to confirm if these differences are associated with the existence of different epitopes.

Hemagglutination properties of this new variant were assessed, though contrasting results were found [209,211]. Nonetheless, agglutination patterns are somehow different from older RHDV strains, predicting changes in the receptors' specificity, which might help to explain differences in pathogenicity [163,209]. The binding site of the new variant to HBGAs was modeled by X-ray crystallography by Leuthold *et al.* [217], and showed that the residues directly interacting with the ABH-fucose of the HBGAs are highly conserved among RHDV strains.

Phylogenetically, the new RHDV variant forms an independent cluster, separated from the pathogenic, older strains (genogroups G1-G6) and from the non-pathogenic strains (Fig. 1.10) [211,214]. Although an outbreak with both the new variant and a G1 strain was recorded [214], it is highly likely that the new variant is replacing the circulating strains due to a competitive advantage [211,214,218]. Evolution from a non-pathogenic virus and a species jump were hypothesized for the emergence of the new RHDV variant [211], but the events leading to the emergence of this new variant are unknown. The remarkable rapid spread of the new RHDV variant, with devastating effects on rabbit

populations [219], highlights the urgent need of the development of strategies to slow down the spread of the virus, namely the development of new vaccines directed specifically towards the new variant.

European brown hare syndrome (EBHS)

In 1980, an unknown epidemic disease, associated with acute liver necrosis, caused death in the European brown hare populations (*Lepus europaeus*, Pallas, 1778) of the Swedish island of Gotland [220]. Given the unknown origin, the disease was called "fältharesjuka" or European brown hare syndrome (EBHS). Its origin remained a mystery for a while and several bacteriological, parasitological and virological hypothesis were considered: an association with a new variety of the oilseed rape (*Brassica napus*, Linnaeus) with low glucosinolate content was hypothesized, but also mycotoxins, selenium deficiency and agricultural pesticides were investigated [220].

The disease rapidly spread in the following years, reaching the southern part of Sweden in the early 1980s. It extended northwards until reaching the 'Nordic limit', a border marking a climate transition and associated with changes in fauna and flora [221]. Denmark followed in reporting the disease, and by 1990s several other European countries also described the same disease (Fig. 1.12) [222-236]. The disease is currently endemic in Europe and was also reported in *L. europaeus* populations introduced in South America [237].

Strikingly, there are indications of the presence of EBHS in Europe several years before the first diagnosis in Sweden, including: (i) descriptions of outbreaks in England from 1976, with hares presenting lesions consistent with EBHS pathology [238]; (ii) Scandinavian hunters knew the disease since the early 1970s; (iii) the virus was detected by PCR in Swedish hare samples collected before 1980 [239]; (iv) since 1962, antibodies against EBHS were detected in archived sera of hares in England [240]. Evidence that EBHS was caused by a virus was found by Lavazza and Vecchi, that observed viral particles similar to RHDV in the liver of dead hares by electron microscopy [233].

Although occurring both in wild and captive hares [220], EBHS is mainly prevalent in free-living hares. Finding carcasses is thus less frequent and the diagnosis of the disease is sometimes problematic due to an advanced state of decomposition [120]. The main reservoir species of EBHS is *L. europaeus*. Infection of mountain hares (*Lepus timidus*, Linnaeus, 1758) also occurs in areas where the distribution range of both species overlaps [221]. European brown hares with chronic forms of EBHS may act as

long-term carriers and shed the virus for long periods, whereas mountain hares die acutely suggesting that the virus could still be adapting to this species [221].

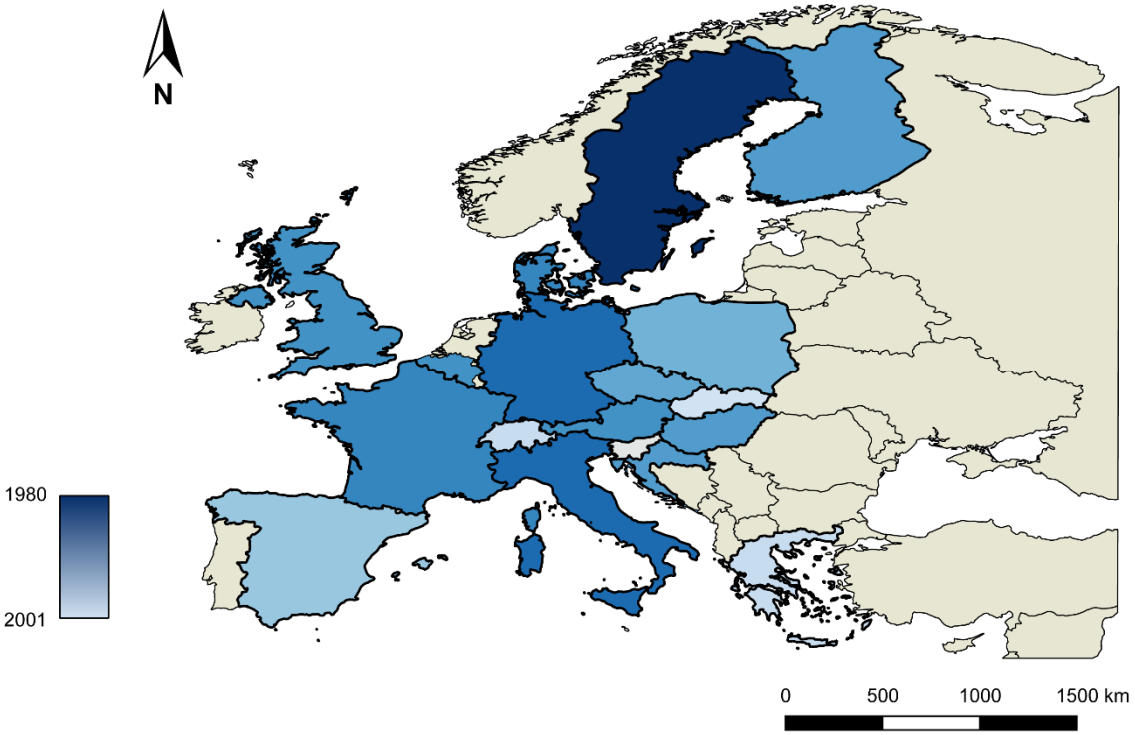

Figure 1.12 Distribution map of European countries reporting EBHS. The blue gradient correlates with the year of the first description of the disease in each country from dark (earlier) to light (later); countries in grey never reported the disease (based in published data only). Adapted from [241].

As for RHD, only adults appear to succumb and young hares (less than 50 days) do not develop EBHS, probably due to the presence of antibodies against EBHS virus transferred through the placenta by passive immunity [220]. The maternally inherited antibodies disappear after weaning. Subadults usually do not develop clinical disease even if infected [112]. Mortality occurs throughout the year but is highest in the fall, between October and December [221]. Unfavorable weather conditions, high population density and concentration of hares in marginal areas after harvesting may account for the highest observed mortality [221]. Additionally, at this time of the year, when hunters are out in the fields, the probability of carcasses to be found is higher, and the young of the year have also lost the maternal protective antibodies, becoming susceptible [221]. Interestingly, in Finland, where *L. timidus* is the main affected species, EBHS cases were reported mainly in spring and summer [242].

When an EBHS outbreak occurs in naïve populations, mortality rates can be up to 100 % in the first two weeks, but in endemic areas mortality rates are low [243]. No

correlation of EBHS susceptibility and sex, matrilinear inheritance or inbreeding coefficient was found [221,222,244]. Following the first outbreak, hare populations seem to reach stability due to protective immunity developed by surviving hares that may either have recovered from the clinical disease or have been infected as young hares [112,245]. In highly endemic areas, the prevalence of antibodies against the virus in wild hare populations can be ~ 90 % [246,247]. Interestingly, a study on farmed hares showed higher antibody titers in 1-3-years old hares, with hares less than one year dying before developing humoral response and old hares not being capable to have a rapid immune response due to reduced stem cell population [244].

The impact of EBHS in European brown hare populations is not easy to assess. Indeed, the population dynamics and their reproductive status are affected by factors other than EBHS, such as fox densities, hunting and agricultural structure [244]. However, low hare densities (less than 8 hares/km^2) are associated with a severe impact of EBHS on the population dynamics [245]. This problem can be addressed by increasing hare densities to ~ 15 adults/km^2 [245]. Hares from areas with high density have higher antibody titers comparing with hares from areas with low density, resulting in a protective effect of the populations [248]. Moreover, in high density areas, the virus circulates endemically, and young hares are in contact with the virus before losing maternal antibodies [249].

EBHS pathology, symptomatology and pathogenicity is similar to RHD [121]. Likewise, and based on the morphology of the liver lesions, several forms of the disease can be recognized. In a peracute form, no clinical signs are visible and hares die quickly. An acute form is characterized by a short clinical course and, after the appearance of the clinical signs, hares die within 2-3 days, but it can last until 7 days post-infection. A subacute or subclinical course may also occur. Hares affected by a chronic form of the disease die after several days or eventually recover [241]. Poor body condition is associated with EBHS infection, having two possible explanations: the disease may cause weight loss or hares with lower weight, caused by poor nutrition, pesticides or other diseases, may have higher susceptibility to infection [221].

Typical characteristics of EBHS infection are the loss of fear of dogs and people, adoption of abnormal postures, jumping into the air, staggering, incoordination and running in circles [243]. Respiratory distress, apathy versus excitement, depression, anorexia and convulsion, probably due to hepatic encephalopathy, are other clinical signs [220,234,238,250]. The liver is the target organ of this disease, but in acute infections gross lesions may be difficult to identify [112]. The diagnosis is sometimes

hampered since other acute infections present the same signs in terminal stages. Furthermore, the chronic form of the disease is only identified by chance, when hares die of other causes [112].

European brown hare syndrome causes generalized hemorrhages and congestion in the inner organs (Fig. 1.13). Liver is usually swollen, pale, yellow, necrotic and with marked lobules. In chronic infections, hares develop jaundice and chronic hepatitis, but the liver presents less extensive necrosis. Hares that recover may have liver fibrosis. Histologically, fatty content change is observed in hepatocytes and hepatocyte necrosis is visible in periportal areas and sometimes in the midzonal areas [e.g. 120,220,251]. Mitochondrial mineralization precedes necrosis and the presence of acidophilic bodies indicates hepatocyte apoptosis [252]. For hares infected experimentally, a decrease in the number of leukocytes and lymphocytes is observed 24h after infection [112]. Before death, a sharp increase of aspartate aminotransferase, alanine aminotransferase, bilirubin and γ-glutamyltranspeptidase is observed at four days post-infection (dpi) [253].

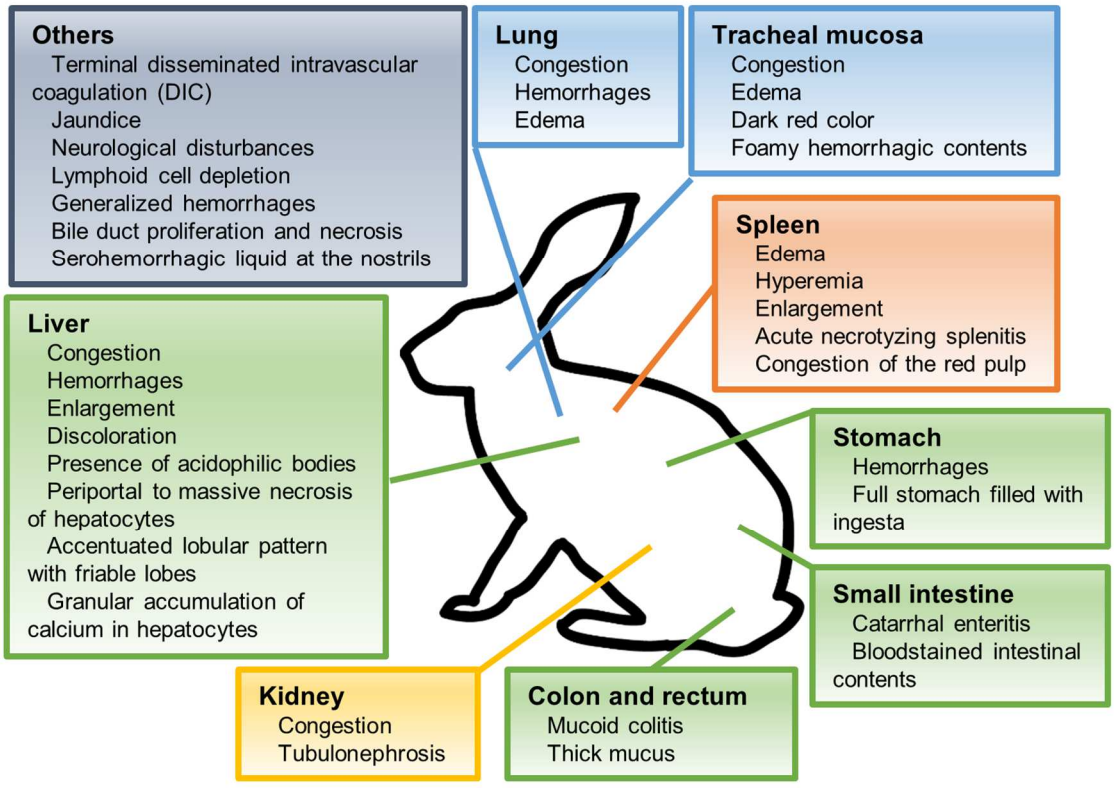

Figure 1.13 Main pathological and histopathological lesions associated with EBHS. Green boxes: lesions in organs of the digestive system; yellow: lesions in organs of the excretory system; orange: lesions in organs of the lymphatic system and blue: lesions in organs of the respiratory system.

This highly contagious disease demands appropriate control measures. Although no commercial vaccines or specific treatments exist for EBHS infection, in farms, vaccines can be produced from livers of hares that died first during the outbreak to immunize the remaining hares [243]. Other containing methods should be applied, including serological tests on new introduced hares, quarantine of these hares and prevention of contact with wild hares and predators by using fencings [243]. Indirect transmission, through contaminated clothes, shoes, grass or hay, should also be avoided. In the wild, eradication is not possible and spread to contiguous naïve populations is difficult to prevent, but translocation of hares to EBHS-free regions should be kept to a minimum. The serological status of the translocated populations should be evaluated and populations must be kept in quarantine with susceptible hares to detected possible incubating or carrier animals [112].

European brown hare syndrome virus (EBHSV)

The viral particles found by Lavazza and Vecchi allowed to identify the similarities between EBHSV and RHDV [233]. The virus was called European brown hare syndrome virus (EBHSV), and later identified as a *Lagovirus* of the family *Caliciviridae* [149]. The biochemical features of EBHSV are typical of the *Caliciviridae* family. Viral particles are small (30-35 nm), icosahedral, with the typical cup-shaped surface depressions of the *Caliciviridae*. This non-enveloped RNA virus has a single major capsid protein of ~ 60 kDa that shares epitopes with the capsid protein of RHDV, showing that these viruses are antigenically related [120,254].

The genome of EBHSV was later cloned and sequenced and has the same organization as RHDV. This positive-sense, single-stranded RNA virus is 7442 base pairs long, but Northern hybridizations showed also the presence of a 2.2 kb subgenomic RNA that probably functions as mRNA for capsid protein synthesis [255,256]. Two slightly overlapping ORFs were determined for EBHSV: ORF1 is the largest and encodes a polyprotein of 2334 amino acids, encompassing the non-structural proteins and the capsid protein, with a predicted molecular mass of 256 kDa, and it shares four cleavage sites with RHDV [256]. The short ORF2 at the 3' end encodes a polypeptide of 114 amino acids and has a predicted molecular mass of 12.4 kDa [255]. A short UTR at the 5' end (8 nucleotides) contrasts with the 92 base pairs long 3'UTR, which is considerably larger than that of RHDV (59 nucleotides). Similarly to RHDV, EBHSV genome has a polyadenylated tail at the 3' end.

Overall, RHDV and EBHSV have 71 % of nucleotide identity and 76 % if considering only the capsid protein gene (Fig. 1.10). Comparison of RHDV and EBHSV capsid proteins revealed a deletion of four amino acids near the N terminus and an insertion of one amino acid near the C terminus of the EBHSV sequence. Interestingly, the highest variability within the capsid is located in the central portion, and it was hypothesized that this central portion is located at the surface of the virion [255]. Further comparison with RHDV showed a similar degree of variation on the non-structural proteins: no major differences on the homology level were observed between structural and non-structural proteins [256,257].

While some authors failed to induce disease in rabbits inoculated with EBHSV (and vice-versa), though a low serological, non-protective, response was observed [120,253,258], others were able to replicate the disease [235,259]. Vaccination of rabbits with EBHSV also gave contradictory results: either EBHSV virus-like particles do not protect against RHDV challenge [260] or induce partial protection [254]. However, experimental infections of cottontails (*Sylvilagus floridanus*) with a virulent EBHSV strain induced mortality [261]. Recently, cottontail populations tested seropositive for EBHSV, albeit with low titers, and one cottontail was found dead during an outbreak of EBHS [262].

EBHSV is a highly contagious virus, preferentially transmitted directly via the oro-fecal route or the respiratory route [241]. Indirect transmission through grass or hay contaminated with excreta or through contaminated clothes or shoes may also occur [112]. Although no reservoir hosts have been yet identified, mechanical vectors may spread the disease. Insects and carnivores are included, as well as birds, since EBHSV was identified in separate islands in Denmark and Sweden [263]. Humans are also possible mechanical vectors, especially hunters that may facilitate virus dissemination. As the virus is very resistant (it resists to pH=3 and inactivation by heat), it may persist several months in the field and still remain infectious.

The lack of a proper culture system to grow the virus *in vitro* impairs its use as a diagnostic tool. Testing the infectivity and isolate the virus is only achieved by experimental inoculation [120]. Alternatively, other methods may be used to establish the cause of death. Diagnostic tests include electron microscopy, PCR, ELISA and hemagglutination [120,220,239]. As the liver is the target organ of EBHSV, it contains the highest viral titer and is the most suitable for virus identification. Due to intense viremia, other organs contain virus particles in a direct proportion to vascularization, thus spleen and serum are also targets for diagnosis. Interestingly, in hares that die from a

subacute or chronic form of the disease, higher levels of subviral particles can be detected in the spleen [241].

Despite the morphological and antigenic similarities between EBHSV and RHDV, the hemagglutination activity of EBHSV is somewhat dissimilar of that of RHDV. In order to hemagglutinate human type O red blood cells, a modified protocol must be performed: the liver homogenate requires a treatment with an equal volume of chloroform, and steps should be carried out at 4 ºC and pH 6.5 [120]. Even using the modified protocol, only 50 % of the samples give positive results. This is likely due to constant lower viral loads when comparing with RHDV and virus degradation, which in turn is probably related to the more frequent subacute and chronic courses of the disease [247].

Few studies have addressed the phylogenetic relationships of EBHSV strains and their evolution. The first phylogenetic reconstruction of EBHSV strains used partial sequences of the capsid protein gene and revealed that old Swedish samples clustered together and apart from other European samples [236]. Other studies evidenced that European samples isolated in the same year were more similar to each other, indicating a relationship with the time of isolation [264-266]. An analysis of French EBHSV samples, using complete sequences of the capsid gene, revealed a correlation between the geographical and the temporal distributions, with older EBHSV strains persisting in the population [267]. These earlier strains coexist with new strains, increasing the genetic diversity. Such pattern contrasts with RHDV evolution, which the authors attributed to not only differences in population densities and absence of vaccination pressure for EBHSV, but also to stronger incidence of the chronic form of EBHS that might facilitate the survival of the earlier strains [267]. Nonetheless, all EBHSV isolates belong to a single serotype.

The origin and emergence of EBHSV and RHDV is difficult to trace. A former presence of non-pathogenic or less pathogenic forms that mutated and acquired virulence was hypothesized [237,240]. This hypothesis is supported by several indications of a former circulation of the viruses [191,238-240,268]. Furthermore, Frölich and colleagues suggested that importation of European hares in the 19th century to South America is associated with virus presence in that continent, that remained apathogenic until a few years ago [237]. Another possible scenario for the emergence of EBHSV and RHDV is a species jump from eastern cottontails (*Sylvilagus floridanus*), imported for recreational activities in the 1960s and the 1970s [269,270]. Despite the lack of knowledge on the emergence of EBHSV, its origin seems to be Scandinavia with a later spread to western, eastern and southern Europe [222].

3. The order Lagomorpha

The order Lagomorpha was recognized in 1912 and is constituted by two extant families: the Ochotonidae (pikas) and the Leporidae (rabbits and hares). Lagomorphs are widespread and very successful [271]. Despite some controversy [see, for example, 272,273], Rodentia and Lagomorpha are usually grouped in the superorder Glires. This grouping was first proposed by Linnaeus in 1758, based on the shared traits between the two orders [272]. The Cretaceous-Tertiary boundary likely marked the split between Glires and other mammals [274,275]. Rodents and lagomorphs started to diverge only at the beginning of the Tertiary (~ 65 million years ago, mya), and are anatomically separated by the number of incisors [271,274]. An Asian origin of Lagomorpha is widely accepted and indeed the oldest known Lagomorpha fossil record is from the early Eocene (~ 53 mya) and was found in central India [276].

The families Ochotonidae and Leporidae are differentiated by the number of teeth (Ochotonidae 26, Leporidae 28) and by the tail and ear length [271]. The two families may have diverged in the early Eocene according to fossil and molecular studies [276,277]. Ochotonids were more diverse during the Miocene, but currently have a single extant genus, *Ochotona*, with 30 recognized species, inhabiting Asia and North America [278,279]. Leporids have 62 extant species distributed by 11 genera and occupy large areas of Eurasia, Africa, North America and Central America. Seven genera are monotypic and have a geographically restricted distribution (*Brachylagus*, *Pentalagus*, *Caprolagus*, *Bunolagus*, *Poelagus*, *Romerolagus* and *Oryctolagus*). Regarding the remaining genera, *Nesolagus* has two species, *Pronolagus* four, *Sylvilagus* 17 and *Lepus* 32 species [279].

The family Leporidae

The phylogenetic history of leporids was assessed in the last decades based on morphological features, analysis of mitochondrial DNA (mtDNA) and chromosomal painting [280-284]. However, Matthee and colleagues [285] pointed out that several constraints, such as the convergence of anatomical features, the incomplete fossil record and the saturation of mtDNA sequences impair a good resolving power of the

phylogenies. Based on a complete analysis of five nuclear and two mitochondrial markers, these authors proposed that the current distribution of this family results from nine dispersal and five vicariance events that occurred mainly during the Miocene and after the origin of the group in North America or Asia (Fig. 1.14) [281,285]. The dispersal events between North America and Asia were facilitated by the formation of land bridges in the Bering strait and the melting of Antarctic ice sheets, at ~ 20 mya [286,287].

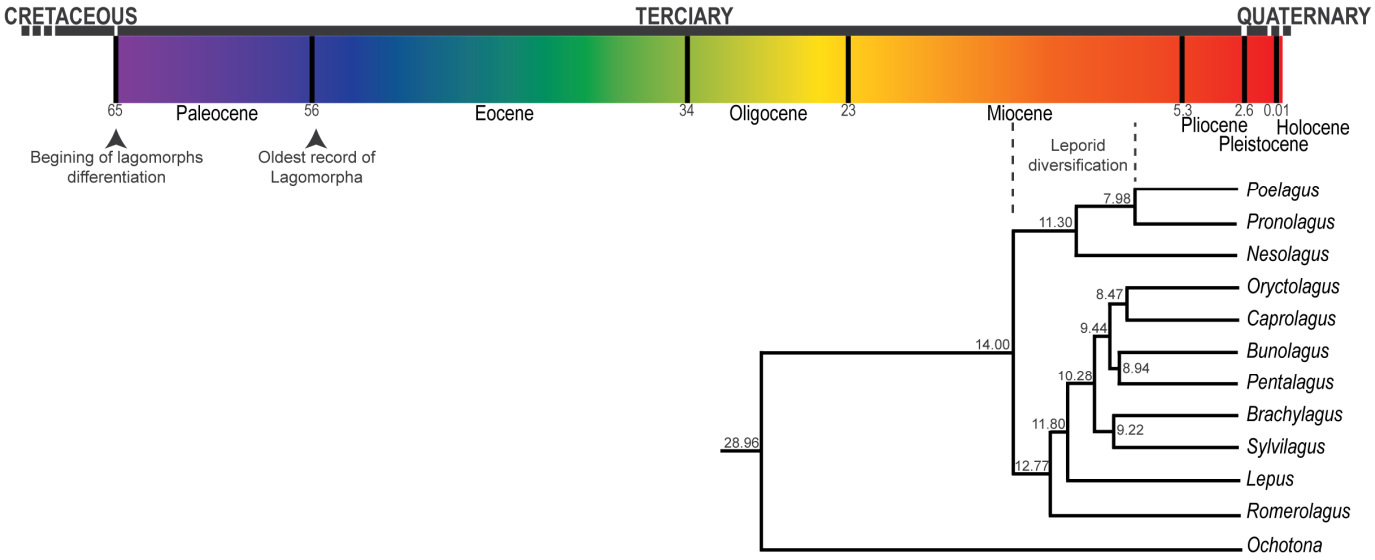

Figure 1.14 Main events in lagomorphs' history and evolutionary relationships among them. In each node of the phylogeny and in the time bar, the time is given in million years. Phylogeny based in data from [285].

Ecological data also support the phylogeny inferred by Matthee and colleagues [285]. Indeed, the basal taxa *Nesolagus* and *Romerolagus* inhabit only regions with dense vegetation, consistent with an ancestral leporid origin in forested environments [288,289]. Moreover, all leporids are obligate herbivores, but the specific diet varies among genera. While the early diverged *Nesolagus* and *Brachylagus*, for example, depend mainly on C_3 plants, the later diverged *Caprolagus* prefers C_4 plants. The expansion of C_4 plants in the late Miocene (~ 7 to 5 mya) and the formation of the Antarctic ice sheet at ~ 6.5 mya are associated with the increased size and wide range expansion of *Lepus*, also facilitated by the development of characteristics suitable for an open habitat [278,285,290].

From the late Miocene until the Pliocene leporids largely differentiated, and expansion to Africa and South America during the Pliocene and Pleistocene promoted the leporid blooming [278]. After the Pleistocene, and due to not only the extreme climate

conditions of the quaternary glaciations but also to the anthropogenic interference, the number of leporid genera decreased substantially [278]. Notwithstanding, leporids are currently widely distributed, occupying the biggest land masses and most islands southeast of Asia. This broad occurrence is largely due to human introductions [271,289], but some introduced species succeeded in new habitats (see, for example, the cases of *Sylvilagus floridanus* in Italy or *Oryctolagus cuniculus* in Australia, that will be further discussed) [130,291].

The European rabbit: a key species versus *a pest*

The fossil records suggest an Iberian origin for the contemporary species of the genus *Oryctolagus*, the species *Oryctolagus cuniculus* [292]. Prior to the Pleistocene ice ages, a southern European distribution is hypothesized [271]. However, during the glaciations, several species had to be confined in southern refugia, such as the Iberian Peninsula [293]. Quaternary glaciations left two main refugia in Iberian Peninsula: one largest refugium in the southwest, close to the Gibraltar strait, and one smaller in the eastern Mediterranean coast [294]. These two refugia were responsible for the differentiation of the two rabbit subspecies that diverged at ~ 1.8 mya, the *Oryctolagus cuniculus algirus* in the southwest and the *Oryctolagus cuniculus cuniculus* in the northeast [295,296].

The species that differentiated in the larger refugium, *O. c. algirus*, has more genetic variability than the species that differentiated in the smaller refugium, *O. c. cuniculus* [296]. After the last glacial maximum, the latter expanded from northern Spain to France, where it still remains present [297]. The difficulties in overcoming the geographical barrier of the Pyrenees created a bottleneck in the gene pool of the individuals and the French wild rabbit population has lower genetic diversity, being a subset of the *O. c. cuniculus* Iberian population [296,297]. Although the subspecies are genetically differentiated, the climatic oscillations (glacial and interglacial periods) in the late Pleistocene allowed contact and admixture [294], and the occurrence of gene flow [295,298]. Currently, *O. c. algirus* and *O. c. cuniculus* share a contact zone from the northwest to the southeast of Iberia [299].

The Portuguese discoveries during the 15th and 16th centuries had repercussions in the rabbit history. Indeed, Portuguese settlers introduced wild rabbits from the subspecies *O. c. algirus* in the archipelago of Azores [300-303]. A strong bottleneck effect is observed in these populations [296]. The *algirus* subspecies is also present in

other Atlantic islands. The *cuniculus* subspecies was introduced in several islands in historical times, as a source of meat for the navigators and colonizers [299]. Human-mediated expansion of rabbits turned them one of the mammals with the most impressive geographical expansion, and colonization was successful all around the world [304].

A founder population of a few hundreds of individuals of the subspecies *O. c. cuniculus* was at the beginning of rabbit domestication. However, the extensive gene flow between the two subspecies before the domestication of rabbits resulted in the introgression of *O. c. algirus* haplotypes in the gene pool of the domestic founder individuals. *O. c. algirus* is therefore an indirect contributor for the domestic gene pool [305]. The origin of domestic rabbits is attributed to French monks that started to domesticate rabbits in the monasteries ~ 1400 years ago [306]. The genetic basis underlying domestication is a subject of interest to many authors, and it was recently shown that domestication was the result of small changes in several genes and not drastic changes in a few genes, leading to the modification of phenotypic characteristics such as the flight response [307]. Due to successive bottlenecks, domestic rabbits present lower genetic diversity than wild rabbits.

In Europe, rabbit is a key species for the ecosystem as several predators, most of them threatened, depend on it to survive. That is the case of the endangered Iberian lynx (*Lynx pardinus*, Temminck, 1827) and of the vulnerable Spanish Imperial eagle (*Aquila adalberti*, Brehm, 1861), both specialist predators on the European rabbit, but other species are affected as well [129,308]. The main cause for the decline of rabbit populations is RHD, although overhunting and habitat loss and fragmentation had also an impact on the population density, even before the emergence of RHD [141,309]. In addition to the ecological impact, a huge economic burden is associated with rabbit decline in Europe, since rabbits are an important game species [310].

While in Europe the conservation of European rabbit is of utmost importance, in Australia rabbits are seen as a pest. As in many other regions of the world, introduction of rabbits was man-mediated and started to occur in the beginning of the 19th century for hunting purposes [130,311]. The pre-existent burrows from native animals helped the rapid dispersal of wild rabbits, at an incredible rate of 70 km/year, slowly leading to the decrease in the numbers and even extinction of native species due to competition for food and shelter [312,313]. The European rabbit is an agricultural pest in Australia and has significantly affected the fauna and flora of that region, changing dramatically the Australian landscape [312,314,315]. Biocontrol measures had to be taken to control the pest, such as the introduction of myxomatosis and later RHD [316]. Rabbits were also

responsible for the extinction of native species in other locations where they were introduced [e.g. 317].

The economic importance of European rabbit also relies on its commercial value as a source of meat and fur. Furthermore, European rabbit is a valuable laboratory model to study several human diseases, as well as toxicology, fertilization and immunology [318-320]. Additionally, myxomatosis and rabbit hemorrhagic disease make rabbits a textbook example to study host-pathogen co-evolution [e.g. 205,321].

The widespread genus Lepus

The cosmopolitan genus *Lepus* probably originated in North America and is the most widely distributed lagomorph [278,285]. In Europe, six hare species are present. The broom hare (*Lepus castroviejoi*, Palacios, 1977) is geographically restricted, occurring only in Cantabrian Mountains, northern Iberia. The Cape hare (*Lepus capensis*) was introduced in Sardinia from Africa, where it has a wider distribution. The Corsican hare (*Lepus corsicanus*) occurs in Italy and in Corsica and the Iberian hare (*Lepus granatensis*, Rosenhauer, 1856) is an Iberian endemic species and covers the whole Peninsula. In the northeast, this species has a narrow contact zone with the European brown hare (*Lepus europaeus*), whose distribution covers all Europe with the exception of the northern part of Scandinavia. Here, *L. europaeus* has a contact zone with the mountain hare (*Lepus timidus*), a species that extends from Scandinavia to the eastern part of Europe, and then has some isolated populations in Ireland, Scotland and in the Alps [279,322].

Although *L. timidus* is not present in Iberian Peninsula, mtDNA lineages of this species were found among *L. granatensis* [323,324]. Similarly, *L. castroviejoi* populations and Swedish and Iberian populations of *L. europaeus* have introgressed mtDNA from *L. timidus* [323,325-327]. Strikingly, no introgressed mtDNA was detected in *L. europaeus* from central Europe [328]. This phenomenon of introgression likely results from ancient hybridization between these species, when *L. timidus* populations were still present in Iberia – the fossil record suggests they became locally extinct at the end of the last glacial period [323,326]. The preservation of these mtDNA lineages allowed them to survive until our days, and considerable frequencies are found in northern and central Iberia. These data further suggest that *L. granatensis* colonized the northern and southern Iberia from a central glacial refugium [reviewed by 329].

Hare population densities are the result of an interplay between abiotic, biotic, anthropogenic and internal factors [330]. Habitat requirements vary along the year and the habitat diversity guarantees food and shelter for the year-round. Hares are more commonly found near arable farming areas, albeit the decrease in hare numbers where intensive agriculture is practiced [331 and references therein]. Nonetheless, European brown hares are adapted to a variety of habitats and were successfully introduced in more exotic regions, like South America, Oceania, Reunion Island and the Falklands [279]. These animals have an important economic role in Europe, where they are considered major game species and thousands of animals are harvested every year [329,332]. On the other hand, the scarcity of European rabbit in some areas in Iberian Peninsula due to viral diseases prompted predators like the Spanish Imperial eagle to choose *L. granatensis* as an alternative prey [129,329].

The introduction of eastern cottontails in Europe

The eastern cottontail (*Sylvilagus floridanus*) is a native American lagomorph, but in the 1950s, 1960s and 1970s this species was successively introduced in Europe, particularly in France and Italy [269,270] for hunting purposes, since rabbit numbers were decreasing because of myxomatosis. At that time, it was expected that cottontails would replace the European rabbit, as myxomatosis is benign in the *Sylvilagus* genus, but that did not occur [333]. In Italy, where it is currently widespread, the eastern cottontail shows a great capacity of dispersal and reproductive performance in the introduced populations. Because of that, the eastern cottontail has occupied the ecological niche of *L. europaeus* [291].

Interspecific competition between *S. floridanus* and the autochthonous species *O. cuniculus* and *L. europaeus* limits these populations and might occur by searching for food and shelter, limited during the winter, by land occupation, or by direct interactions between individuals [291]. For the latter, body size (higher in hares, successively followed by rabbits and cottontails) is an important factor determining the success of aggressive interactions [334]. In areas where eastern cottontails and European brown hares are sympatric, the introduced species is pointed as the causative agent of the declining of hare populations [335]. Moreover, the interactions between this introduced species and the others present in Europe need attentive investigation, because of the possible role of eastern cottontail in acting as a reservoir of viral diseases and in their transmission [336].

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Chapter 2

Insights into the emergence and evolution of the new RHDV variant in Portugal

Paper I. Abrantes J, Lopes AM, Dalton KP, Melo P, Correia JJ, Ramada M, Alves PC, Parra F, Esteves PJ: **New variant of rabbit hemorrhagic disease virus, Portugal, 2012-2013**. *Emerging Infectious Diseases* 2013, **19**:1900-1902.

Paper II. Lopes AM, Correia J, Abrantes J, Melo P, Ramada M, Magalhães MJ, Alves PC, Esteves PJ: **Is the new variant RHDV replacing genogroup 1 in Portuguese wild rabbit populations?** *Viruses* 2015, **7**:27-36.

Paper III. Lopes AM, Dalton KP, Magalhães MJ, Parra F, Esteves PJ, Holmes EC, Abrantes J: **Full genomic analysis of new variant rabbit hemorrhagic disease virus revealed multiple recombination events**. *Journal of General Virology* 2015, **96**:1309-1319.

Paper IV. Almeida T, Lopes AM, Magalhães MJ, Neves F, Pinheiro A, Gonçalves D, Leitão M, Esteves PJ, Abrantes J: **Tracking the evolution of the G1/RHDVb recombinant strains introduced from the Iberian Peninsula to the Azores islands, Portugal**. *Infection, Genetics and Evolution* 2015, **34**:307-313.

New variant of rabbit hemorrhagic disease virus, Portugal, 2012–2013

J. Abrantes, A. M. Lopes, K. P. Dalton, P. Melo, J. J. Correia, M. Ramada, P. C. Alves, F. Parra & P. J. Esteves

1. To the Editor: During November 2012–February 2013, rabbit hemorrhagic disease virus (RHDV) strains belonging to the new variant RHDV were isolated in Portugal from wild European rabbits (*Oryctolagus cuniculus* subsp. *algirus*). The major capsid protein, VP60, of these strains was partially characterized. RHDV had been previously been detected in Portugal in 1989 [1]. Before 2011, RHDV outbreaks in wild European rabbit (*O. cuniculus*) populations in the Iberian Peninsula were exclusively caused by strains belonging to genogroup 1 [2,3].

In the Iberian Peninsula, 2 subspecies of European rabbit are found, *O. cuniculus* subsp. *algirus* and *O. cuniculus* subsp. *cuniculus*. These subspecies are equally susceptible to RHDV [3]. In 2011, a new variant was isolated in young rabbits belonging to *O. cuniculus* subsp. *cuniculus* from a rabbitry in the province of Navarra, Spain [4]. The topology of the variant's phylogenetic tree and the susceptibility of kits < 2 months old suggest that this strain is similar to that described in France in 2010 [5].

Before the new variant of RHDV emerged and, on the basis of phylogenetic relationships, RHDV strains had been divided into 6 genogroups (G1–G6) [1], with strains of G6, or RHDVa, having a distinct antigenic profile [6]. All of these strains replicate in the liver and are responsible for causing death in rabbits > 2 months of age. Non-pathogenic and weakly pathogenic RHDV-related strains have also been described. The non-pathogenic and weakly pathogenic strains are phylogenetically distinct from the G1–G6 strains with ~ 20 % of nucleotide divergence [7]; they typically replicate in the intestines [8,9]. New variant RHDV causes death in kits as young as 30 days old and affects vaccinated and unvaccinated animals [4]. Phylogenetically, this new variant falls between the non-pathogenic groups [4,5].

During November 2012–February 2013, our laboratory, CIBIO, Universidade do Porto, Portugal, received liver samples from wild adult rabbits and kits, belonging to *O. cuniculus* subsp. *algirus*, from three areas of Portugal, Valpaços, Barrancos, and Algarve. The rabbits had appeared dead and had clinical signs suggesting rabbit hemorrhagic disease (RHD). We analyzed the samples for RHDV by reverse transcription PCR. For this process, total RNA was extracted by using the RNeasy Mini Kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions. Reverse transcription was performed by using oligo(dT) as primer (Invitrogen, Carlsbad, CA, USA) and SuperScript™ III Reverse Transcriptase (Invitrogen) as recommended by the manufacturer. Screening of the samples consisted of PCR with a pair of primers as described by Dalton *et al.* [4]. This pair amplifies a 738-bp fragment of the gene encoding the capsid protein, VP60 (PCR conditions are available on request). After purification, PCR products were sequenced on an automatic sequencer ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems, Foster City, CA, USA) with the same pair of primers.

The virus was detected in 15 samples, five from each locality. The obtained sequences were aligned with those available from public databases. Retrieved sequences represent the RHDV groups G1–G6, the non-pathogenic groups, and the new variant (GenBank accession nos. KF442960–KF442964). A phylogenetic tree was inferred in MEGA5 [10] by using a Maximum Likelihood (ML) approach. Reliability of the nodes was assessed with a bootstrap resampling procedure consisting of 500 replicates of the ML trees. The best-fit nucleotide substitution model was determined by using MEGA5.

Our sequences exhibit the highest nucleotide sequence identity with the RHDV N11 strain from Spain (99 %; GenBank accession no. JX133161.1), which corresponds to the new RHDV variant. Thirteen nucleotide substitutions were detected in comparison to the Spanish sequence, 3 of which were non-synonymous. The inferred ML phylogenetic tree is in agreement with those published [1,3,9]. G1–G6 (pathogenic) RHDV strains and non-pathogenic and weakly pathogenic RHDV-related strains (generally referred to as RCV) form 2 groups (Fig. 2.1). The non-pathogenic strain from Australia (RCV-A1_Australia_MIC-07) does not cluster with other non-pathogenic groups and European brown hare syndrome virus (EBHSV_France) appears in a basal position in the tree. As described, the new variant (N11_Spain) appears between RCV and the non-pathogenic Australian strain [4,5]. The strains isolated from rabbits in Portugal cluster with the new variant and form a highly supported group (bootstrap value 1.00). These results support the conclusion that the virus recovered in Portugal belongs to the new variant RHDV described in Spain and France.

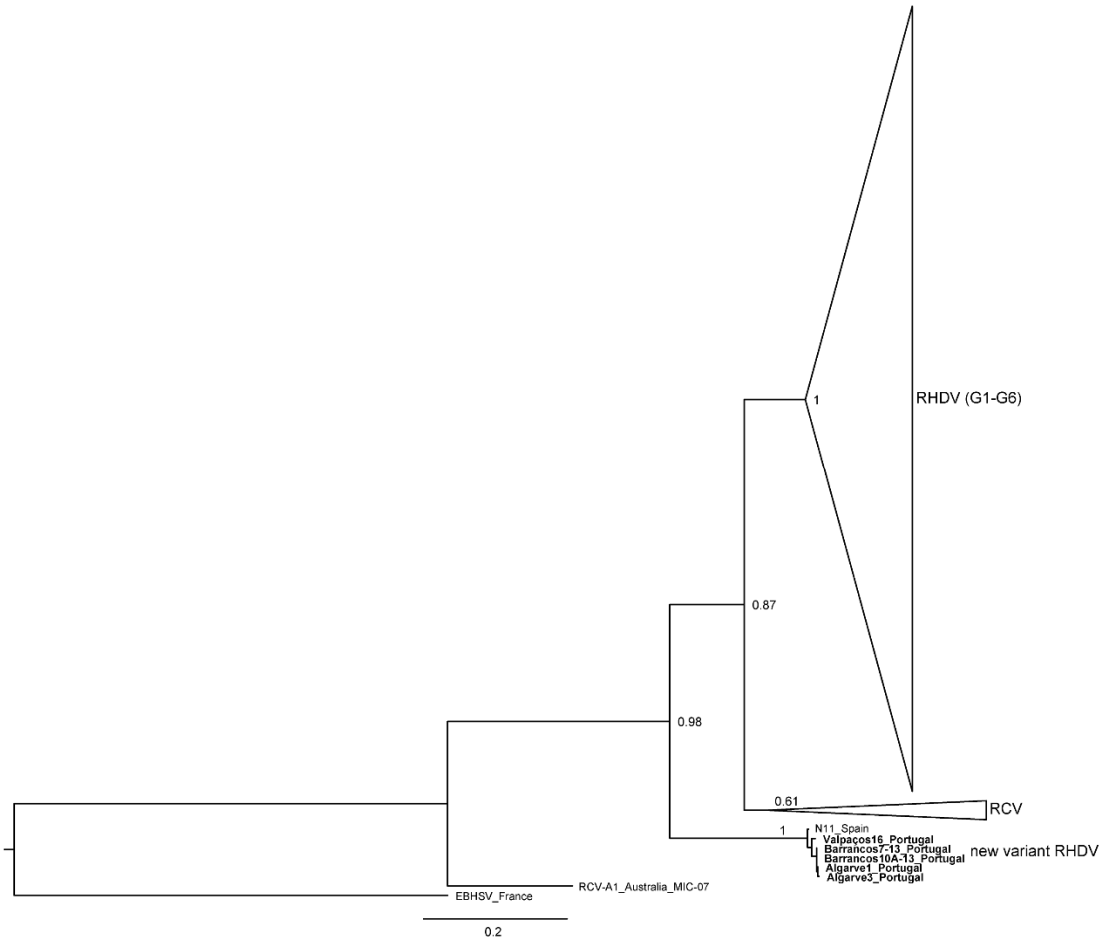

Figure 2.1 Maximum likelihood phylogenetic tree of 95 partial sequences of the rabbit hemorrhagic disease virus (RHDV) capsid gene. Bootstrap values appear next to the nodes and are shown only for the major groups: G1–G6 (GenBank accession nos. AB300693, AF231353, AF258618, AF453761, AJ006019, AJ302016, AJ303106, AJ495856, AJ535092, AJ535094, AJ969628, AM085133, AY269825, AY523410, AY926883, AY928268, AY928269, DQ069280, DQ069281, DQ069282, DQ189077, DQ189078, DQ205345, DQ280493, DQ530363, DQ841708, EF363035, EF558572, EF558573, EF558574, EF558575, EF558576, EF558577, EF558578, EF558581, EF558582, EF558583, EF558584, EU003578, EU003579, EU003580, EU003581, EU003582, EU250330, EU650679, EU650680, FJ212322, FJ212323, FJ794179, FJ794180, FN552800, FR823354, FR823355, GU339228, GU373617, GU373618, GU564448, HE963222, HM623309, HQ917923, JF412629, JF438967, JN165233, JN165234, JN165235, JN165236, JN851729, JN851730, JN851731, JN851732, JN851733, JN851734, JN851735, JQ815391, JQ995154, L48547, M67473, RHU49726, X87607, Y15424, Y15427, Z24757, Z29514, Z49271), RCV (GenBank accession nos. GQ166866; AM268419; X96868) and new variant RHDV (GenBank accession no. X133161). European brown hare syndrome virus (EBHSV) was used to root the tree (GenBank accession no. NC_002615). A non-pathogenic strain from Australia was also included (GenBank accession no. EU871528). The samples isolated from the rabbits found in Portugal appear in bold (Valpaços16_Portugal, Algarve1_Portugal, Algarve3_Portugal, Barrancos7-13_Portugal, Barrancos10A-13_Portugal, GenBank accession nos.: KF442960–KF442964. Scale bar indicates nucleotide substitutions per site.

This confirms the presence of the virus in wild rabbits on the Iberian Peninsula. We also confirm that both European rabbit subspecies are susceptible to the new variant. The appearance and rapid spread of the new variant RHDV into the Iberian wild rabbit populations raise concern for the survival of these populations in this region. These

conservation concerns are particular highlighted for the *O. cuniculus* subsp. *algirus*, because it only occurs in the southwestern part of the Iberian Peninsula, and it is a key prey species for several carnivores, namely, for the most endangered feline, the Iberian Lynx (*Lynx pardinus*). Therefore, monitoring the spread and evolution of this new variant is crucial in determining the most appropriate conservation measures.

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Is the new variant RHDV replacing genogroup 1 in Portuguese wild rabbit populations?

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1. Abstract

The *Lagovirus* rabbit hemorrhagic disease virus (RHDV), a member of the family *Caliciviridae*, severely affects European rabbit (*Oryctolagus cuniculus*) populations by causing rabbit hemorrhagic disease (RHD). RHDV is subdivided in six genogroups but, more recently, a new RHDV variant with a unique genetic and antigenic profile emerged. We performed a study in rabbits found dead in the field during 2013 and 2014 in Portugal to determine the prevalence of this new variant *versus* the classical RHDV. Fifty-seven liver samples were screened for the presence of RHDV and positive samples were genotyped. All cases of RHDV infection were caused by the new variant. The only former genogroup circulating in Portugal, G1, was not detected. We hence conclude that the new RHDV variant is replacing G1 in Portugal, probably due to a selective advantage. This sudden and rapid replacement emphasizes the necessity of continued monitoring of wild rabbit populations.

2. Introduction

Rabbit hemorrhagic disease virus (RHDV), a member of the genus *Lagovirus*, family *Caliciviridae*, is responsible for causing rabbit hemorrhagic disease (RHD). RHD was first reported in China in 1984 [1] and is a highly contagious and fatal disease for European rabbit (*Oryctolagus cuniculus*). The acute form of the disease can cause up to 80 – 100 % of mortality within 1–3 days [2,3]. RHD is associated with hepatic necrosis, hemorrhages and congestion in several organs, including the upper respiratory tract and lungs, and splenomegaly [2]. The disease became endemic in several European countries, where it was first reported in 1986 [4], and in Australia and New Zealand,

where it was introduced as biological control agent [5]. In Portugal, RHD was described in 1989 [reviewed in 3].

RHDV is a non-enveloped, positive-sense, single-stranded RNA virus, with a genome of ~ 7.4 kb organized in two ORFs, with ORF1 encoding the major structural capsid protein gene, VP60. Historically, six RHDV genogroups were recognized based on the VP60: genogroups 1–5 (G1–G5) and the antigenic variant RHDVa or G6 [6,7]. However, more recent studies showed that G3, G4 and G5 belong to the same genetic cluster [8,9]. Furthermore, weak pathogenic and non-pathogenic forms of the virus exist, which are genetically different from the pathogenic RHDV, exhibiting ~ 20 % divergence [10-14]. The other member of the genus *Lagovirus*, the closely related European brown hare syndrome virus (EBHSV), has a similar organization of the genome, albeit presenting ~ 30 % divergence from RHDV [15].

In 2010, a new RHDV variant emerged in France [16], tentatively named RHDV2 or RHDVb, that rapidly spread to several European countries including Portugal, where it was described in 2012 [17-21]. With a unique antigenic profile, it causes mortality in young rabbits (less than two months) and vaccinated rabbits, that are typically not susceptible to G1–G6 strains [17]. Vaccinated rabbits appear to be less susceptible to this new variant, albeit protection conferred by the vaccine is not complete [19]. Before the emergence of the new variant, only G1 strains were known to circulate in Portugal [22-24]. RHDVa was also described in the Iberian Peninsula [25], but this description refers only to domestic rabbits and no reported cases exist in wild animals.

The Iberian Peninsula is inhabited by two subspecies of *O. cuniculus*: one occupying the northeastern part (*O. c. cuniculus*) and the other restricted to the southwest and the islands of Madeira, Azores and Canaries (*O. c. algirus*) [reviewed in 26]. Both subspecies are equally affected by RHDV [22], but the susceptibility of the subspecies *O. c. algirus* acquires special importance since its distribution is restricted to the Iberian Peninsula. Thus, this subspecies is more vulnerable to severe outbreaks, which might have implications on its conservation status.

In this work, we performed a genetic study to determine the prevalence of the new variant comparing to G1 in Portuguese rabbit populations and a histopathological study to characterize the lesions found in young rabbits. Our results confirm that the new variant has replaced G1 in Portugal in the last two years, possibly reflecting a selective advantage.

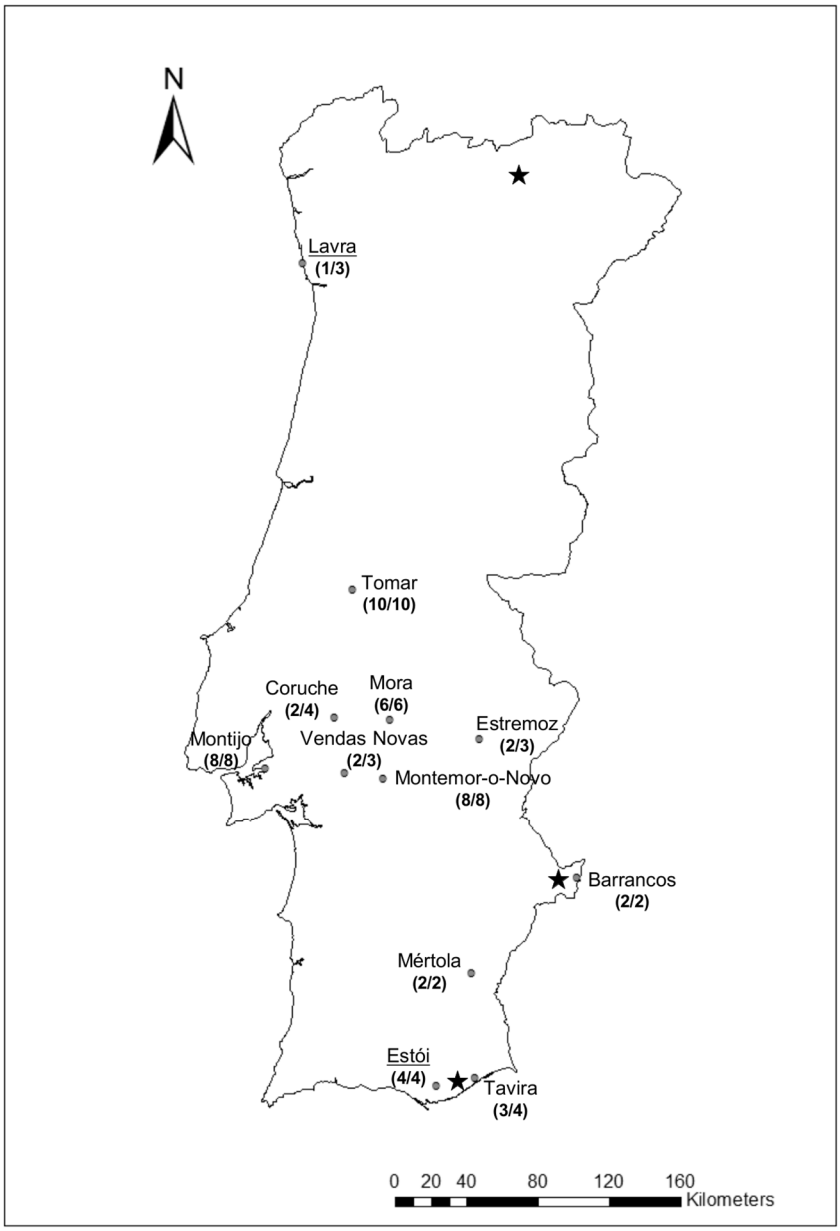

Figure 2.2 Map of Portugal with sampling localities indicated. Localities sampled in 2013 appear underlined; in parenthesis, the number of positive samples per total number of samples is shown. The approximate locations of the first descriptions of the new variant's presence in Portugal according to [18] are marked by the black stars.

3. Methods

Fifty-seven liver samples were collected in 12 localities covering from north to south of Portugal, between 2013 and 2014 (Fig. 2.2), and frozen at −20 °C. All rabbits were found dead in the field with lesions consistent with RHD and, based on the geographical distribution, belong to the subspecies *O. c. algirus* [26]. In addition, for seven young wild rabbits, carcasses were submitted to necropsy and samples of lung, liver, thymus, trachea, kidney and spleen were collected for histopathological exam. Tissue samples were fixed in 10 % buffered formalin and embedded in paraffin. Sections

of 3 µm thick were stained with hematoxylin and eosin for routine microscopical examination.

Total RNA was extracted from the liver of all samples with the RNeasy Mini Kit (Qiagen, Hilden, Germany). Up to 30 mg of liver were collected and tissue was homogenized in a rotor-stator homogenizer (Mixer Mill MM400 from Retsch, Haan, Germany) at 30 Hz for 7 min. RNA extraction followed the manufacturer's instructions. Viral cDNA was synthesized using oligo(dT) primers and SuperScript™ III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. A screening to test for the presence of RHDV was performed with two pairs of primers: the first pair is specific for the new variant: RHNavF 5'-GTTCGTCAAATGTACTTGAGC-3' and RHNavR 5'-GTGTACGTAATGGCACTACTG-3' [27]. Samples that tested negative were screened with a second more general pair of primers (RHDV4831F 5'-GTGTATGCCATGACTCCGAT-3' and RHDV_VP60_0467R 5'-GCGTCGATGACAACATGAG-3'). These primers were used in previous studies and successfully amplified several RHDV strains including G1, RHDVa and G3–G5 like [24,25,28,29]. For positive samples, the complete capsid protein gene, VP60, with 1740 base pairs, was amplified (PCR primers and conditions are available upon request). After purification, PCR products were sequenced on an automatic sequencer ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems, Foster City, CA, USA) with the amplification primers. These sequences were deposited in GenBank with the accession numbers KM115667-KM115716 and aligned in BioEdit version 7.0.9.0 [30]. Each sequence was compared and blasted against publicly available sequences in NBCI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). In order to facilitate visualization and the subsequent analyses, a maximum of ten sequences of the main RHDV groups were used (G1–G6, the new variant, non-pathogenic RCV-A1 and weakly pathogenic MRCV; EBHSV was also included). Genetic distances between our samples and each RHDV group were calculated in MEGA6 (p-distance method, 1000 replicates) [31]. Additionally, a Maximum Likelihood (ML) tree, using a representative of each unique haplotype found, was estimated using the same software. The best-fit nucleotide substitution model as determined in MEGA6 (GTR+G+I) and a bootstrap resampling analysis of 1000 replicates, were used to reconstruct the phylogeny.

4. Results and Discussion

Between 2013 and 2014, Portuguese wild rabbit populations suffered severe RHD outbreaks. Indeed, during that period, we received in our laboratory rabbit samples from

several Portuguese localities (Fig. 2.2) that were shown to be positive for the new RHDV variant. This new variant was described in Portugal in 2012 [18]. Interestingly, before that, G1 was the only genogroup known to be circulating in Portugal [22-24].

While the macroscopic and histopathological alterations caused by RHDV are well-known, little is known on how the new RHDV variant affects wild animals. As this is a new and emergent virus, monitoring these alterations might give insights on eventual changes in lesions. At necropsy, the alterations and histopathological lesions observed were similar to those observed for other animals infected with the new RHDV variant and that are typical of a RHDV infection [16,17,19]. Necropsy of the seven young rabbits revealed epistaxis, hemorrhagic tracheitis with sero-hemorrhagic exudate in the lumen of the trachea, sero-hemorrhagic pleurisy, pulmonary congestion (predominantly in the dorsal region), and multiple hemorrhages scattered throughout the organs. Livers were predominantly pale in appearance and in some cases were congested. The thymus, spleen and kidneys showed also congestion. Histopathology revealed hemorrhagic pneumonia and tracheitis, congestion of the liver and diffuse necrotizing hepatitis (Fig. 2.3A,B). This first establishment of a diagnosis and consequent cause of death was then confirmed by the molecular techniques.

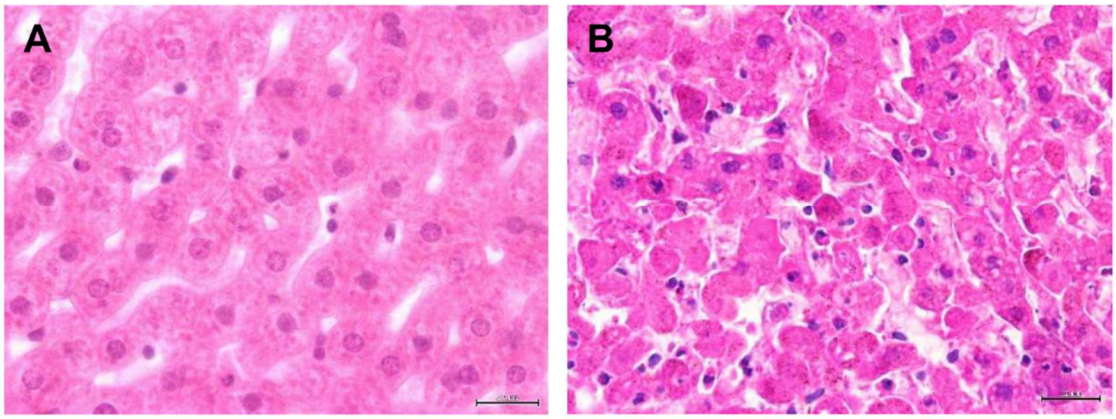

Figure 2.3 Histopathological characterization of the new rabbit hemorrhagic disease virus (RHDV) variant in Portuguese samples. (**A**) Liver section of a healthy rabbit. Staining H&E, scale bar = 20 µm; (**B**) Liver with lesions of microvacuolar degeneration and necrotizing hepatitis with karyolysis. Staining H&E, scale bar = 20 µm.

Of the 57 rabbit liver samples, 50 were positive for the new variant (87.7 %; Fig. 2.2); all but one were successfully amplified for the entire capsid gene. The 143 nucleotide differences defined 33 unique haplotypes, with an overall identity of 98.7 % (± 0.1%). None of the samples was positive for G1 or for any other genogroup. The blast analysis revealed 96 – 98 % of identity with publicly available sequences of the new variant from Italy and France (GenBank accession numbers KC741409, HE800529-32,

FR819781, HE819400, JQ929052, KC345611-13, JX106022-23, KC907712), and an overall identity of 82 % with G1 strains. The analysis of the nucleotide identities between our dataset and genogroups showed an identity of 81.6 % (± 0.8 %) with G2, 82.1 % (± 0.8 %) with RHDVa and 82.2 % (± 0.8 %) with G3-G5. Furthermore, our sequences presented a nucleotide identity of 81 % (± 1.0 %) with the weakly pathogenic MRCV, 79.2 % (± 0.8 %) with the non-pathogenic RCV-A1 (strain MIC-07) and 70.0 % (± 0.9 %) with EBHSV (overall identity with strains O4021-9, BS89 and EBHSV-GD; GenBank accession numbers of these sequences are indicated in Fig. 2.4). The ML tree also confirmed that all samples belong to the new RHDV variant group (Fig 2.4), since all RHDV samples recovered from Portuguese rabbits cluster with publicly available sequences described as belonging to the new RHDV variant (bootstrap value of 99; Fig. 2.4). Comparison with the partial sequences of the capsid obtained in the first reported cases of the new RHDV variant in Portugal (GenBank accession numbers KF442960-64) [18], revealed that our sequences present between 0.0 % (± 0.2 %) and 2.5 % (± 0.6 %) divergence. However, the small length of the analyzed fragment does not allow us to make any conclusions about the epidemiology and dispersal of the virus since the initial outbreaks.

Indeed, the lack of epidemiological data on RHDV prevalence in Portuguese wild rabbit populations impairs a precise comparison between G1 and the new variant virulence/pathogenicity. However, the number of dead animals found in the field in 2013 and 2014 seems to have substantially increased, as has been described in Spain by Delibes-Mateos et al [32]. Our results show that these RHD outbreaks were caused by the new variant and no apparent cases of G1 (or other genogroup) were detected. This indicates that the new variant is the main variant currently circulating in Portugal and has potentially replaced G1; the factors contributing to this replacement are still unclear, but are currently under study. A similar situation has been described in France and Spain, where the new RHDV variant appears to be replacing G5 and G1, respectively [19,33,34].

After the first description of the new variant in Portugal in three Portuguese localities in 2012 and 2013 [18], the new RHDV variant was able to disperse to the center and south of Portugal (Fig. 2.2), suggesting a high capacity of dispersal in a very short period of time. The weak dispersal to the north of Portugal is possibly due to low rabbit densities. By contrast, in the south, the high densities of rabbits facilitate virus dispersal. The high capacity of dispersal, associated with the replacement of G1, suggests a selective advantage of the new variant over other genogroups, possibly by overcoming existing immunity to older strains. Le Gall-Reculé and colleagues [19] reported that the

new RHDV variant is less virulent than classical RHDV in experimental conditions and that this could be advantageous for the new variant, by giving more time to the virus to spread. The different mortality rates observed between studies [e.g., 17,19] make possible another scenario, where the new variant is still evolving to acquire its optimal virulence. Another possibility is that the new RHDV variant might be evolving towards higher levels of virulence, as observed in a recent study in Australia, where RHDV virulence has been increasing due to the development of genetic resistance in rabbits in Australia [35].

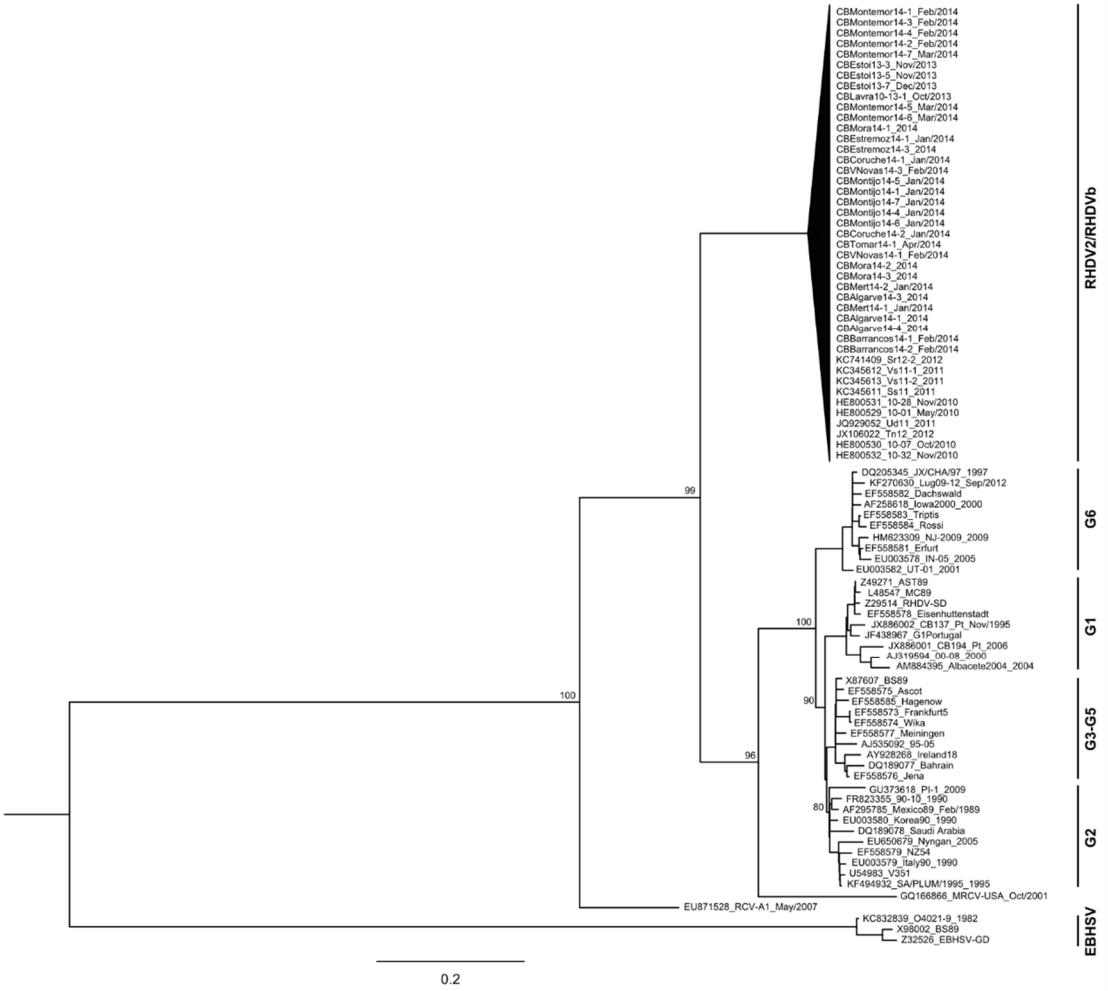

Figure 2.4 Maximum Likelihood tree of the capsid gene. Bootstrap values of the main nodes (≥ 80) are shown next to them. To facilitate visualization, the new variant group is collapsed (previously identified new variant strains have their accession numbers indicated). Samples were named according to the sampling locality, the year of collection and the number of the isolate (cf. Fig. 2.2); when known, month and year of collection are indicated. Scale bar indicates the nucleotide substitutions per site. GenBank accession numbers of the publicly available sequences used in this study are indicated.

The threat presented by the new RHDV variant to the European rabbit might be particularly high for *O. c. algirus*, since distribution of this subspecies is limited to the southwest of Iberian Peninsula. In addition, the presence and circulation of the new RHDV variant compromises the reintroduction of the most endangered felid in the world (Iberian lynx, *Lynx pardinus*) in Portugal, as rabbits represent 90 % of its diet. The European rabbit is also economically important as game species, being a traditionally coveted prey, with high social value. Since the epidemiological outcome of G1 replacement by the new variant is unpredictable, and the new variant is causing important ecological and economical losses in Portugal, rabbit translocations should also be highly restricted, and continued monitoring of natural rabbit populations is strongly recommended.

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Full genomic analysis of new variant rabbit hemorrhagic disease virus revealed multiple recombination events

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1. Abstract

Rabbit hemorrhagic disease virus (RHDV), a *Lagovirus* of the family *Caliciviridae*, causes rabbit hemorrhagic disease (RHD) in the European rabbit (*Oryctolagus cuniculus*). The disease was first documented in 1984 in China and rapidly spread worldwide. In 2010, a new RHDV variant emerged, tentatively classified as 'RHDVb'. RHDVb is characterized by affecting vaccinated rabbits and those < 2 months old, and is genetically distinct (~ 20 %) from older strains. To determine the evolution of RHDV, including the new variant, we generated 28 full-genome sequences from samples collected between 1994 and 2014. Phylogenetic analysis of the gene encoding the major capsid protein, VP60, indicated that all viruses sampled from 2012 to 2014 were RHDVb. Multiple recombination events were detected in the more recent RHDVb genomes, with a single major breakpoint located in the 5' region of VP60. This breakpoint divides the genome into two regions: one that encodes the non-structural proteins and another that encodes the major and minor structural proteins, VP60 and VP10, respectively. Additional phylogenetic analysis of each region revealed two types of recombinants with distinct genomic backgrounds. Recombinants always include the structural proteins of RHDVb, with non-structural proteins from non-pathogenic lagoviruses or from pathogenic genogroup 1 strains. Our results show that in contrast to the evolutionary history of older RHDV strains, recombination plays an important role in generating diversity in the newly emerged RHDVb.

2. Introduction

Rabbit hemorrhagic disease (RHD) is an acute fatal necrotizing hepatitis that affects wild and domestic European rabbits of both the *Oryctolagus cuniculus cuniculus* and *Oryctolagus cuniculus algirus* subspecies [1]. The disease was first detected in 1984 in China following the importation of commercially bred Angora rabbits from Germany [2]. RHD rapidly spread worldwide and is currently endemic in several countries including Portugal, Spain and France. The economic burden of RHD on rabbit farming and hunting industries and the negative ecological impact on the wild rabbit populations and in their dependent predators, particularly in the Mediterranean ecosystem, are well known and of major concern [3]. In contrast, in Australia, RHD has been successfully exploited as a form of biocontrol to reduce rabbit numbers, although there is mounting evidence for host resistance [4-6].

RHD is caused by rabbit hemorrhagic disease virus (RHDV), a *Lagovirus* of the family *Caliciviridae*. RHDV has a non-enveloped single-stranded (ss) RNA genome organized into two narrowly overlapping open reading frames (ORFs). ORF1 encompasses nucleotide residues 10–7044 and encodes a large polyprotein that is cleaved by a virus encoded protease to generate several non-structural (NS) proteins and the major capsid protein, VP60 or VP1 (for convenience, we use VP60). ORF2 comprises nucleotide residues 7025–7378 and produces VP10 (or VP2), a minor structural protein [7]. At the 5' region, the RHDV genome presents a covalently linked protein, VPg, and is polyadenylated at the 3' region [8,9]. Viral particles also package an abundant subgenomic RNA (sgRNA) that contains the coding sequences of VP60 and VP10. As is the case for the RHDV genomic RNA, the sgRNA is VPg-linked and polyadenylated [9-11].

Despite the importance of RHDV for animal health and ecosystem well-being, its origin and emergence as a pathogenic virus in the European rabbit is not well understood. On current data the most likely explanation is direct evolution from a non-pathogenic form of the virus [12]. Indeed, a number of non-pathogenic and a weakly pathogenic rabbit caliciviruses have been described in Europe, America and Australia [13-16], and there is evidence for the existence of RHDV as a non-pathogenic form before the first documented outbreak [reviewed by 1]. To date, however, the mutations (or recombination events) responsible for the profound change in virulence are unknown. It is also theoretically possible that a cross-species transmission event was central to the emergence of RHDV as a pathogenic form. Indeed, in the 1990s, RHDV affected a species other than the European rabbit [17].

More recently, a new variant of RHDV was detected in France [18]. As a definitive RHDV nomenclature has not yet been agreed (various terms such as ‘new variant’, ‘RHDVb’ and ‘RHDV2’ have been used), for simplicity we use the term ‘RHDVb’. RHDVb resulted in atypical RHD outbreaks in that it led to mortality in both vaccinated adult rabbits [18] and young rabbits [19,20] that are typically resistant to RHDV. Phylogenetic relationships inferred from the VP60 gene showed that RHDVb formed a novel phylogenetic group that fell between the RCV-like viruses, i.e. European non-pathogenic viruses and the weakly pathogenic Michigan rabbit calicivirus (MRCV) and the Australian non-pathogenic rabbit caliciviruses (RCV-A1) [18]. Further work demonstrated that RHDVb is antigenically different from other RHDVs [19,21], although its genesis is uncertain. Importantly, the recent detection of RHDVb in leporid species other than the European rabbit [22,23] indicates that it might infect a wider range of host species.

Both host jumping and changes in virulence have been associated with the ability of RNA viruses to rapidly produce genetic diversity through mutation [24,25]. However, it is also possible that the genotypes associated with successful emergence are generated by recombination [26]. Because it requires template switching, (homologous) RNA recombination generally involves regions of high sequence similarity. Although homologous recombination has been detected in RHDV [27,28], its impact on fitness and hence long-term importance remains uncertain. Notably, the RHDV strain isolated in 1984 in China had a recombinant origin [28], which may have been in part responsible for the marked change in virulence observed in this virus.

RHDV has been divided into six genogroups, denoted G1–G6 [29]. In the Iberian Peninsula, RHDV is characterized by a high degree of virus isolation with only G1 detected in wild rabbit populations [30-32], which differs from the epidemiological pattern seen in other European countries [29]. However, in 2011 and 2012, RHDVb was detected in both Spain and Portugal [19,33]. To better understand the evolution of RHDV and particularly the occurrence and impact of recombination, we determined the complete coding sequences of Iberian RHDV strains collected between 1994 and 2014.

3. Methods

Virus samples and genome amplification

Liver samples were collected from wild rabbits found dead in the field in different regions of Portugal and from domestic rabbits from Spanish rabbitries, and were kept

frozen at -20 ºC. No live animals were shot, trapped or handled to obtain tissues; as such, no animal ethics permit was required. Each frozen liver was thawed and up to 30 mg of the liver was collected. The tissue was homogenized in a rotor-stator homogenizer (Mixer Mill MM400; Retsch) at 30 Hz for 7 min. Total RNA was extracted using the RNeasy Mini kit (Qiagen) according to the protocol provided by the manufacturer. Reverse transcription was performed using oligo(dT) as primers and SuperScript™ III Reverse Transcriptase (Invitrogen) according to the provided protocol. The complete genome of each RHDV strain was obtained by the amplification of several overlapping fragments ranging from ~ 700 to 1700 bp using 1 µL cDNA reaction mix, 2 pmol of each oligonucleotide, 5 µL Phusion Flash High-Fidelity PCR Master Mix (Thermo Scientific) and water to a final volume of 10 µL. The primers and PCR conditions used are summarized in Table 2.1. For recombinant sequences, each PCR was performed at least twice independently. After purification, PCR products were sequenced on an automatic sequencer ABI PRISM 310 Genetic Analyzer (Applied Biosystems). Sequences were determined using the amplification primers and are available in GenBank.

No incongruences were detected between the overlapping fragments that could indicate preferential amplification of templates in each PCR. While it is highly unlikely that the Phusion DNA Polymerase generates crossovers at the same position repeatedly, a long range PCR was also performed to obtain complete genomic sequences and exclude the possibility of PCR-mediated artefacts in generating the recombinant sequences. This PCR was conducted with the following conditions: 0.25 µL of Takara LA *Taq* (Clontech), 2.5 µL buffer, 2.5 mM $MgCl_2$, 2.5 mM of each dNTP, 0.4 µM of each primer, 1 µL of template and water to a final volume of 25 µL. Cycle conditions consisted of 2 min at 94 ºC, 35 cycles of 30 s at 94 ºC, 30 s at 63 ºC and 8 min 30 s at 68 ºC and a final extension of 15 min at 68 ºC. The amplicons were sequenced as described above using amplification primers listed in Table 2.1 (primer 5'-GTGTATGCCATGACTCCGAT-3' covers the regions located both upstream and downstream of the recombination breakpoint).

Recombination analysis

The complete genome sequences obtained (excluding the 5' and 3' UTRs) were aligned using the BioEdit software, version 7.0.9.0 [34]. Full-length RHDV genome sequences available in public databases were retrieved and included in the alignment. This produced a final dataset of 68 sequences of 7369 nt, including representatives from all major groups of RHDV. This sequence alignment was screened for recombination

using the RDP, GENECONV and BootScan methods implemented in the RDP software (version 4.26) [35] under the following parameters: sequences were set to linear, Bonferroni correction, highest acceptable *p*-value 0.05 and 100 permutations. Only recombination events detected by all three methods were considered as viable and hence merited additional analysis (see *Phylogenetic analysis* section). Strains detected as recombinants were further analyzed using the SimPlot software (version 3.5.1) [36] with the default parameters (window size, 200 bp; step, 20 bp; Ts/Tv ratio, 2 : 0).

Table 2.1 List of primers and PCR conditions used in the amplification of the RHDV genome. Ta: annealing temperature.

Forward primer (5'-3')	Reverse primer (5'-3')	Ta (ºC)	Extension time
GTGAAAGTTATGGCGGCTATGTCG	TTCCCAAGGACCATGGTGTGT	55	1 min 30 s
AGGTGCACCCTGCCATCATACAA	ATGAGCGTGGTCTGTGAGTGGAA	53	1 min 15 s
CAACATCTTTGGCGCATGGT	TACGCCAGCACGTCAATCTT	51	1 min
GCAAACCACCTTGTCAACCT	ATGGCGTCAAGTATGGTCATGG	48	1 min 30 s
GTTGGCGTTGACATGACATC	CTAGTAGTGGCCACAACACC	52	1 min 15 s
GTGTATGCCATGACTCCGAT	GCGTCGATGACAACATGAG	48	1 min 30 s
TGCTGAGCCAGATGTACGCT	AGTCCAATTGTCACTGGCAG	52	1 min 15 s
CACCTGTGGGTAAGAACACA	ATAGCTTACTTTAAACTATAAACCCAA	50	45 s

Phylogenetic analysis

An initial reconstructed phylogenetic tree was inferred for the VP60 gene to provide a provisional picture of the evolutionary relationships of RHDVb to the remaining pathogenic and non-pathogenic lagoviruses. For this analysis, all available VP60 sequences were retrieved from public databases, producing a final dataset of 211 sequences of 1740 nt. A phylogenetic tree was then estimated using the ML method available in PhyML program [37] utilizing the GTR+I+Γ model of nucleotide substitution and employing a combination of nearest-neighbor interchange (NNI) and subtree pruning and regrafting branch-swapping. The support for each node was determined from 1000 bootstrap replicate ML trees, employing the same substitution model and parameter values as above, and with NNI branch-swapping. For the complete genome sequences, the dataset was partitioned at the putative recombination breakpoint(s) detected in the recombination analysis described in the Results and Discussion section and then subjected to phylogenetic analysis. This partitioning was as follows: (i) ORF1 except VP60 (nucleotide residue positions 10–5304) and (ii) VP60 and VP10 (nucleotide residue

positions 5305–7378). Phylogenetic trees were then estimated for both partitions using the same phylogenetic procedure as described above (i.e. ML trees in PhyML).

4. Results and Discussion

We determined the complete coding sequence (excluding the 5' and 3' UTRs) of 28 RHDV strains from rabbits found dead in the Iberian Peninsula. Based on their position in the Maximum Likelihood (ML) tree of the capsid gene (VP60) (Fig. 2.5), 24 strains were identified as RHDVb and four as genogroup 1 (G1). Considering the distribution of the European rabbit subspecies in the Iberian Peninsula [38,39], these results further confirm that both *O. cuniculus algirus* and *O. cuniculus cuniculus* are susceptible to this new virus [33]. Interestingly, the co-circulation of RHDVb and other RHDV genogroups, particularly G1, was not detected. This supports the notion that G1 has been replaced by RHDVb [40] and is compatible with the rapid spread of this new variant through the Iberian Peninsula [20].

The complete coding sequences of RHDVb exhibited some length variation due to deletions in the non-structural proteins p16/NS1 and p37/NS3: a deletion of codon 68 (p16) in CBAnd1/Spain/04-2012 and deletions of codons 134–135 (p16/NS1) and of codon 714 (p37/NS3) in several strains. While the biological role of p16/NS1 is unknown, a recent study of RHDV in Australia revealed a relatively high number of mutations in this protein, which were tentatively associated with increased virulence [41]. Protein p37 has an ATPase activity and may be a member of the helicase superfamily III [42]. Although helicase activity is crucial for replication [43], this deletion is located some distance from the Walker motifs A (amino acid positions 522–529 in RHDV) and B (amino acid positions 566–567 in RHDV) which are typical of NTP-binding proteins, and from an additional conserved C motif (amino acid positions 600–614 in RHDV), which is also involved in ATP hydrolysis [42]. This indicates that the main biological functions of p37 are not compromised by the deletion. Additionally, the deletion is present in MRCV, RCV-A1 and in European brown hare syndrome virus (the other member of the genus *Lagovirus*), further suggesting that the replication activity of p37 is maintained. A mutation in the initiation codon (T to C in codon ATG, nucleotide positions 7025–7027) of ORF2 was observed in one strain, but expression of VP10 is not expected to be affected as translation of ORF2 relies on the presence of a sequence element at the 3' end of ORF1 and not on a particular initiation codon [44]. Additionally, another ATG in this region (nucleotide positions 7037–7039) may act as the start site for translation of ORF2.

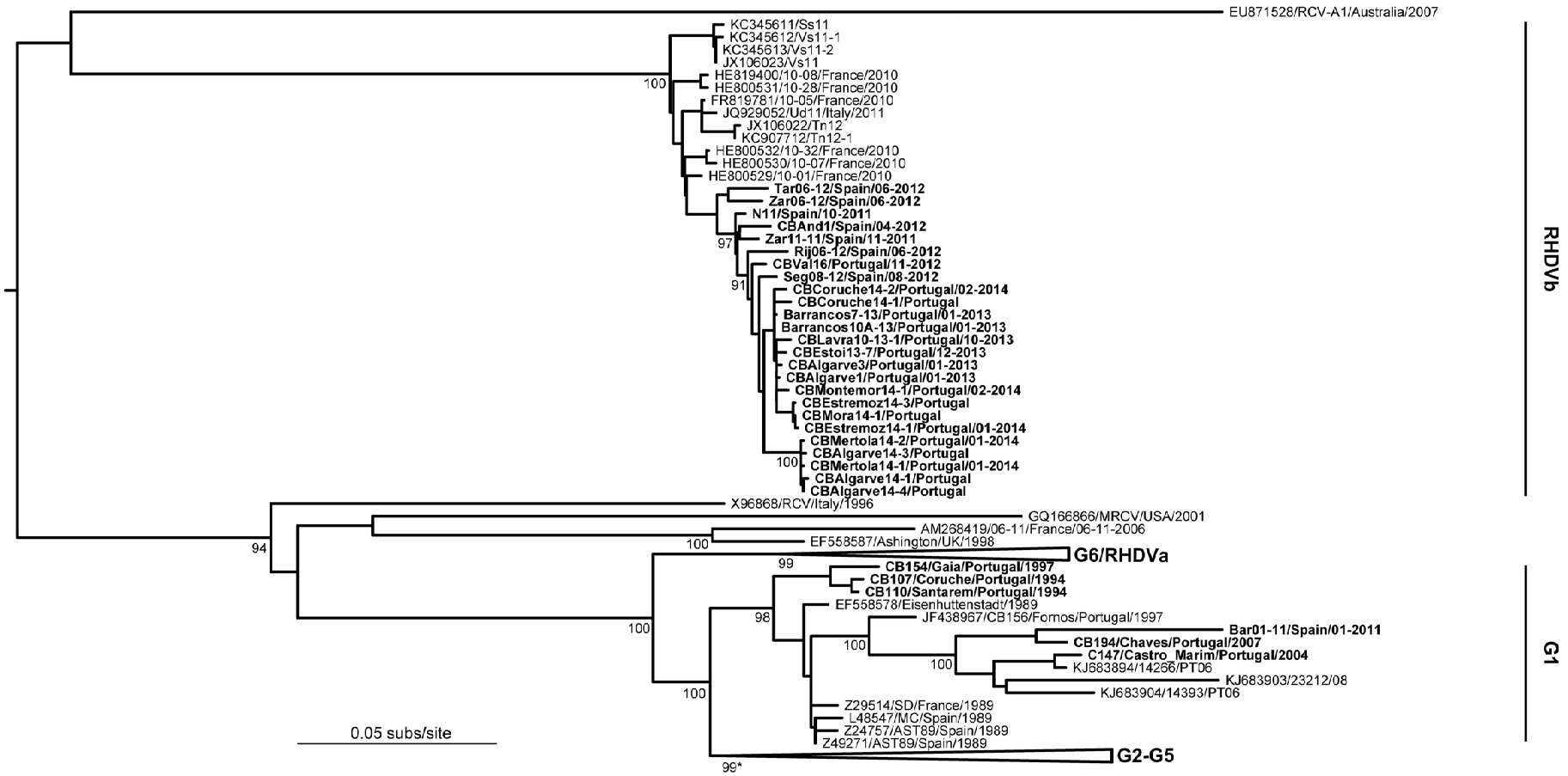


Figure 2.5 ML phylogenetic tree for 211 VP60 sequences of RHDV. Major genetic groups (genogroups) are indicated. For clarity of presentation, the sequences in genogroups G2–G5 and G6 (RHDVa) have been collapsed and have the following GenBank accession numbers: AB300693, AF231353, AF258618, AJ006019, AJ302016, AJ303106, AJ495856, AJ535092, AJ535094, AJ969628, AM085133, AY925210, AY926883, AY928268, AY928269, DQ069280–DQ069282, DQ189077, DQ189078, DQ205345, DQ841708, EF363035, EF558572, EF558574, EF558575, EF558577, EF558581–EF558584, EU003578–EU003582, EU250330, EU650679, EU650680, FJ212322, FJ212323, FJ794179, FJ794180, FN552800, FR823354, FR823355, GU339228, GU373617, GU373618, GU564448, HE963222, HM623309, HQ917923, JF412629, JN165233–JN165236, JN851729–JN851735, JQ815391, JQ995154, JX393309–JX393312, KC345614, KC595270, KF270630, KF494906–KF494952, KF537692, KF537693, KF594474–KF594476, KF677011, KJ579156–KJ579160, KJ606958, KJ606959, KJ683893, KJ683900, KJ683902, KJ683905–KJ683908, KJ814617–KJ814622, M67473, RHU49726, RHU54983, X87607, Y15424 and Y15427. The accession numbers are indicated for the remaining sequences. Viruses sequenced here (in bold) fall into two distinct clusters, G1 and RHDVb. All horizontal branch lengths are drawn to a scale of nucleotide substitutions per site and the tree is mid-point rooted. Bootstrap values greater than 75 % are shown for key nodes. *The main group of sequences in genogroups G2–G5 clustered together with 99 % bootstrap support.

Table 2.2 Results of the recombination analysis.

Recombinant strains	Most likely 'parental' strain		Genogroup		Breakpoint†	Average *p*-value		
	NS*	VP60 and VP10	NS*	VP60 and VP10		RDP	GENECONV	BootScan
CBAnd1/Spain/04-2012 CBMontemor14-1/Portugal/02-2014 CBEstoi13-7/Portugal/12-2013 CBMora14-1/Portugal CBEstremoz14-1/Portugal/01-2014 CBEstremoz14-3/Portugal CBLavra10-13-1/Portugal/10-2013 CBAlgarve1/Portugal/01-2013 CBAlgarve3/Portugal/01-2013 Barrancos7-13/Portugal/01-2013 Barrancos10A-13/Portugal/01-2013 CBCoruche14-1/Portugal CBCoruche14-2/Portugal/02-2014	EU871528/RCV-A1/Australia/2007	Seg08-12/Spain/08-2012	NP‡	RHDVb	5305 (5267-5326)	4.272×10^{-125}	2.898×10^{-132}	2.971×10^{-137}
CBMertola14-2/Portugal/01-2014 CBMertola14-1/Portugal/01-2014 CBAlgarve14-1/Portugal CBAlgarve14-3/Portugal CBAlgarve14-4/Portugal	JX886001/CB194/Chaves/Portugal/2006	Seg08-12/Spain/08-2012	G1	RDHVb	5336 (5264-5343)	6.283×10^{-109}	3.610×10^{-106}	6.316×10^{-109}

*Non-structural proteins.

†99% confidence interval is indicated.

‡Non-pathogenic strain.

The most striking result of our study was the occurrence of recombination in a number of recent RHDVb viruses with strong statistical support (*p*-values < 0.001, Table 2.2). In these recombinant viruses, there was consistent evidence for a single recombination breakpoint located in a region close to the VP60 initiation codon (although two recombination breakpoints were detected they fall in very similar and overlapping locations, which is strongly suggestive of a single recombination event; Table 2.2). This was further confirmed with the SimPlot analysis (Fig. 2.6). Notably, this recombination breakpoint is located within a region of high sequence similarity (data not shown) and divides the genome into two regions that include different protein subsets: one that includes the non-structural proteins and another that comprises the structural proteins VP60 and VP10. To confirm this recombinant history, ML phylogenetic trees were inferred for the different genome fragments on either side of the recombination breakpoint identified above. For simplicity, we based this phylogenetic analysis on the recombinant breakpoint at nucleotide position 5305. For the phylogenetic tree of the genes encoding the structural proteins (VP60 and VP10 genes; Fig. 2.7a), all viruses previously identified as RHDVb (Fig. 2.6) clustered together in a single strongly supported monophyletic group (identified as RHDVb and shaded light grey). This cluster fell deep in the VP60 and VP10 phylogeny, close to the position of the mid-point root of the tree and between the non-pathogenic Australian strain (RCV-A1) and the weakly pathogenic MRCV. Hence, RHDVb is phylogenetically distinct from the pathogenic RHDV sequences that form a separate highly supported cluster. In contrast, in the phylogeny inferred for the non-structural proteins (Fig. 2.7b), the RHDVb sequences fall into three distinct clusters: (i) one comprising likely non-recombinant RHDVb sequences (shaded light grey) that share a common ancestor with pathogenic RHDV strains, (ii) another composed of likely recombinant RHDVb sequences that cluster with MRCV and RCV-A1 (medium grey) and (iii) a group of RHDVb recombinant sequences that cluster closely with recently sampled pathogenic G1 viruses (dark grey).

The occurrence of multiple recombination events, i.e. RHDVb clusters (i) and (ii) mentioned above, makes it difficult to clearly distinguish recombinants from parents, but it is clearly necessary to invoke at least two independent recombination events involving RHDVb to explain these phylogenetic data. Moreover, the divergent position of the CBAnd1/Spain/04-2012 sequence within the RHDVb cluster (ii) may be indicative of an additional recombination event. It is also evident that one of the recombination events involved the combination of the RHDVb VP60 and VP10 coding sequences with a backbone composed of the coding sequences of the non-structural proteins of a G1-like strain, while in the other the RHDVb VP60 and VP10 coding sequences became linked

with the coding sequences of the non-structural proteins of non-pathogenic lagoviruses (Fig. 2.7b).

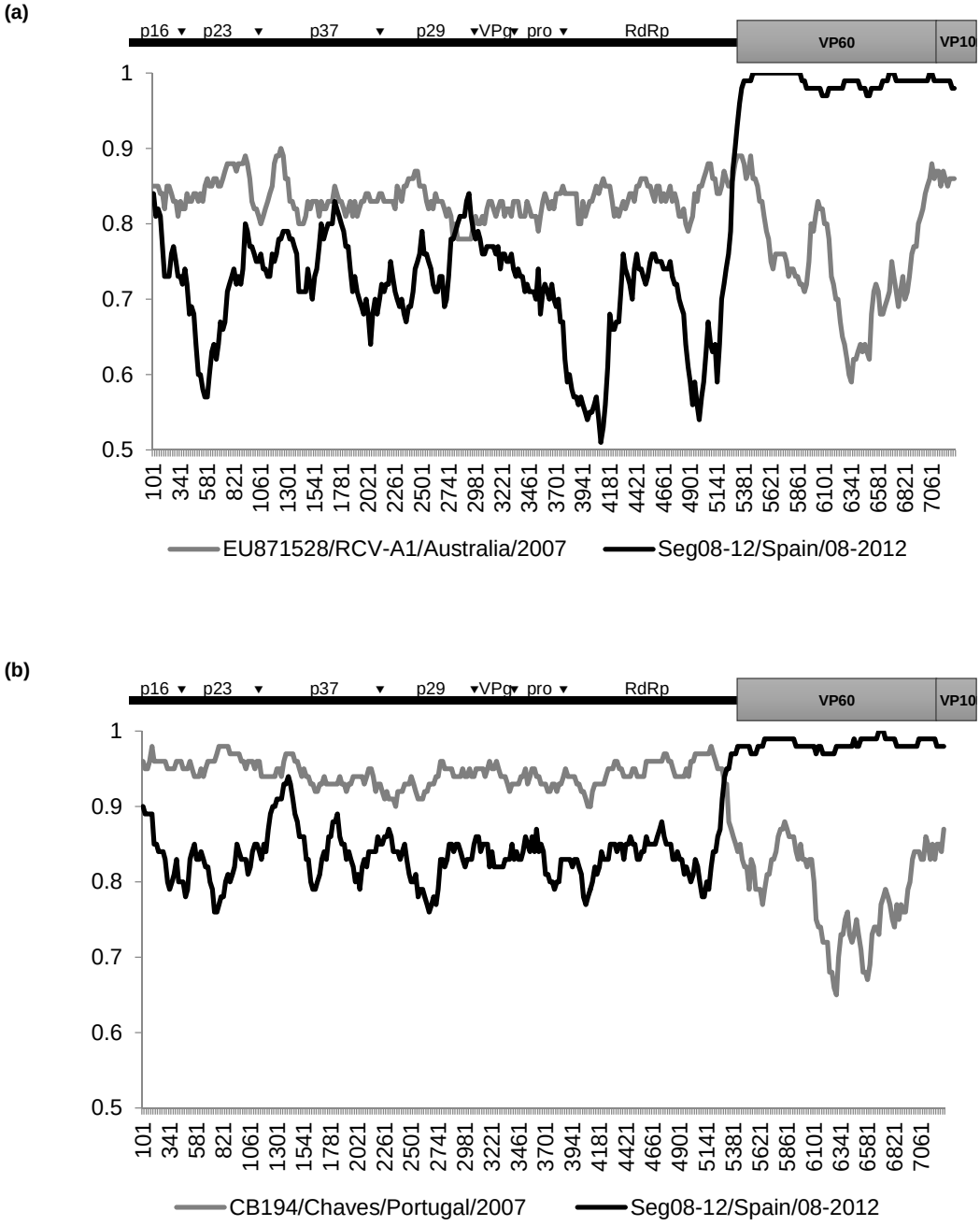


Figure 2.6 Similarity plot for two types of RHDVb recombinants. (a) CBAlgarve1/Portugal/01-2013 (non-pathogenic lagovirus/RHDVb) and **(b)** CBAlgarve14-1/Portugal (G1/RHDVb). The vertical axis represents the percentage of sequence identity of each putative parental strain (grey and black lines) with the recombinant. The horizontal axis indicates the nucleotide positions. A window size of 200 bp with a step size of 20 bp was used. The site where the parental strains are identical in sequence to the recombinant is the predicted recombination breakpoint. A scheme of the genomic organization is shown above each plot with the encoded proteins indicated (pro, protease); arrowheads indicate cleavage sites.

Since some of the recombinant viruses possess non-structural genes that are closely related to the Australian non-pathogenic virus (as supported by the clustering of such recombinant viruses with RCV-A1 with 98 % bootstrap support), this evolutionary history implies that lineages related to the Australian viruses must circulate in the Iberian Peninsula but have not yet been detected (indeed, it is clear that RCV-A1 was imported into Australia [45]). In addition, recombinant sequences were retrieved from animals found dead in the field with lesions compatible with RHD, and thus these strains are likely pathogenic despite their genome deriving mostly from non-pathogenic viruses.

(a)

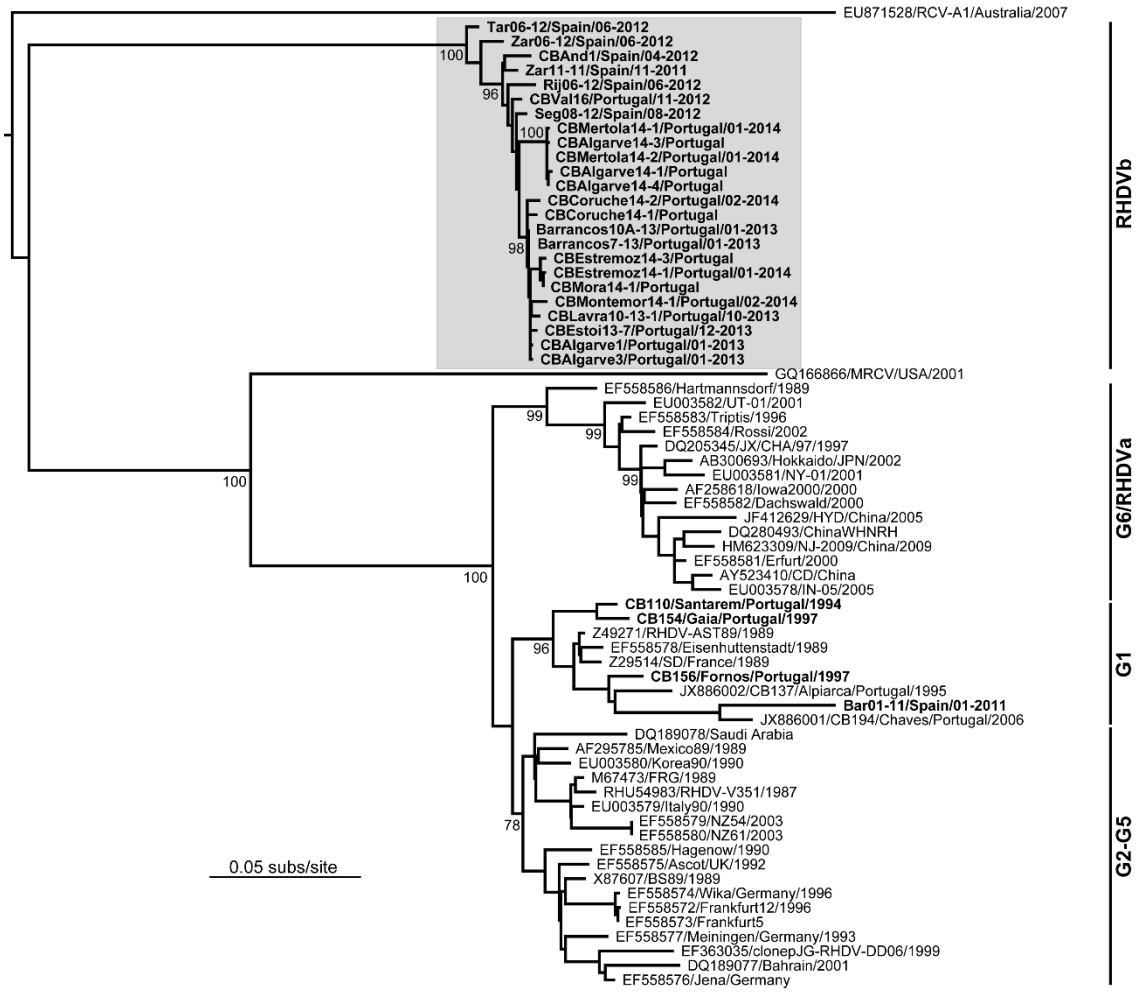
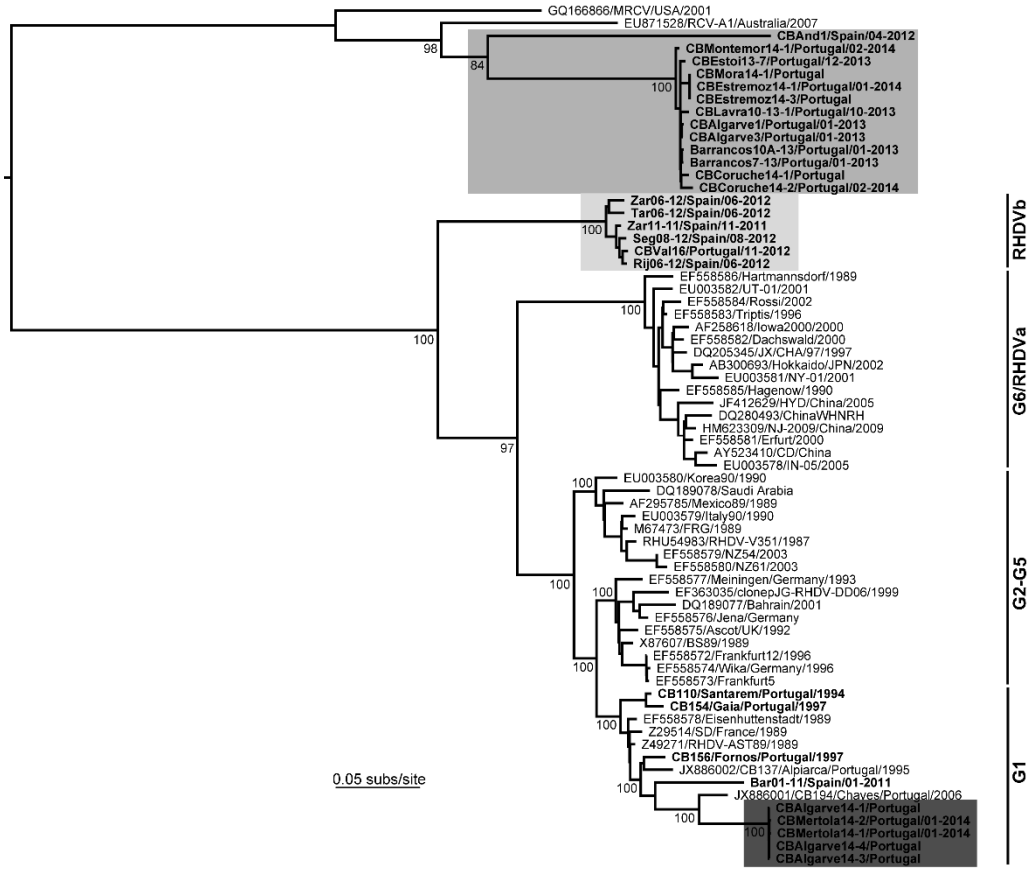


Figure 2.7 ML phylogenetic trees for the genome regions defined by the recombination analysis. Major genetic groups (genogroups) are indicated and strains sequenced here are shown in bold. Accession numbers of the sequences retrieved from GenBank are indicated. All horizontal branch lengths are drawn to a scale of nucleotide substitutions per site and the tree is mid-point rooted. Bootstrap values greater than 75 % are shown for key nodes. **(a)** ML tree based on the sequence fragment including VP60 and VP10 (nucleotide positions 5305-7378). RHDVb sequences are shaded light grey. **(b)** ML tree based on non-structural proteins sequences (nucleotide positions 10-5304). RHDVb strains fall into three distinct clusters corresponding to: (i) non-recombinant RHDVb sequences (shaded light grey), (ii) non-pathogenic lagovirus/RHDVb recombinants (medium grey) and (iii) G1/RHDVb recombinants (dark grey).

Figure 2.7 (cont.)

(b)



Although circulation of 'true' G1 viruses has not been detected in this study from 2011 onwards, the co-circulation of G1-like and RHDVb strains within the same geographical region and time-frame must have occurred for recombination to take place. Interestingly, the two different types of recombinants were detected from the same geographical area only one year apart (e.g. CBAlgarve14-1/Portugal, detected in January 2014, and CBAlgarve1/Portugal/01-2013, detected in January 2013). These observations indicate that there is a relatively high diversity of RHDV strains circulating in this region, which clearly merits additional surveillance. In addition, the same type of recombinant was observed in several and distantly located populations and in different years, confirming its viability and capacity to spread at the epidemiological scale.

Two mechanisms of RNA recombination are possible for RNA viruses: replicative and non-replicative. The (copy-choice) replicative mechanism involves template switching by the viral polymerase during RNA synthesis. In other *Caliciviridae* genera, such as *Sapovirus*, *Vesivirus* and *Norovirus* (NoV), a recombination hotspot is similarly observed between the non-structural proteins and the capsid junction [46]. In the case

of NoV, a template switching mechanism has been proposed that reflects a combination of the copy-choice and the internal initiation model [47]. Accordingly, recombination occurs in the second round of replication when the polymerase starts the synthesis of a positive-sense RNA strand from a full-length negative strand. Due to the presence of complex secondary structures, such as stem–loops, at the ORF1/ORF2 overlap that coincides with the RNA promoter sequence, the polymerase loses processivity and switches to a negative strand of sgRNA synthesized by a co-infecting virus, thereby creating a hybrid virus [47]. Alternatively, the polymerase switches from a genomic RNA to another in regions with high sequence homology, such as the one found where ORF1 and ORF2 overlap [47]. Although the genomic organization of NoV differs from RHDV with the non-structural proteins and the capsid protein encoded by separate ORFs, the synthesis of the sgRNA by an internal initiation mechanism and the presence of a subgenomic promoter upstream of the sgRNA starting site in RHDV [9] suggests that a similar recombination mechanism may occur. In addition, the presence of a region of high sequence homology upstream of the initiation codon of VP60 in RHDV [48], which is compatible with the location of the sgRNA promoter [9,49-51], as well as the presence of a stem–loop [48], tentatively suggest that the recombination events observed could have been due to the template switching mechanism.

However, it is also possible that the recombinant RHDV genomes observed might have been generated by a non-replicative recombination mechanism. Such a mechanism, demonstrated for other positive-sense ssRNA viruses such as polioviruses and pestiviruses [52,53], involves the cleavage of the recombining RNAs that are then ligated to form the recombinant molecule. In contrast to the template switching mechanism of recombination that is promoted by sequence conservation, the non-replicative recombination seems to be largely mediated by the presence of a RNA secondary structure [54] such as the stem–loop predicted for RHDV in the sgRNA promoter region [48].

While the impact of recombination on the fitness of RHDVb remains to be assessed, this process has clearly created substantial changes in the viral genome, although whether this has led to differences in virulence is unclear. Indeed, the recombination events described involve the combination of structural and non-structural protein subsets with different ancestries: protein coding sequences are combined as ‘modules’ with non-structural proteins originating either from non-pathogenic forms or G1 viruses and structural proteins originating from RHDVb. A similar model of modular viral evolution has been described for enteroviruses (family *Picornaviridae*), in which the structural and non-structural regions of the genome evolve semi-independently [55-57],

giving rise to new virus variants. RNA signals and sequence similarity are among the factors that might impose such a recombination pattern [58], although other constraints exist at several stages of viral infection and replication in the host. These constraints most likely reflect functional protein 'blocks' that work together for protein compatibility. Interestingly, previous studies revealed recombination breakpoints throughout the RHDV genome [27,28], although most of these recombination events occurred between closely related RHDV strains. Hence, it is likely that the sequence similarity between the recombining strains allows the functional integrity to be maintained. As there is prior evidence that recombination may have been central to the emergence of RHDV as a pathogenic form [28], we contend that more effort should be directed toward revealing the frequency and fitness consequences of recombination in lagoviruses.

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Tracking the evolution of the G1/RHDVb recombinant strains introduced from the Iberian Peninsula to the Azores islands, Portugal

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1. Abstract

Previous genetic characterization of rabbit hemorrhagic disease virus (RHDV) from Azores, Portugal, revealed the presence of genogroup 3–5 (G3–G5) *like* strains. These strains differed from the genogroup 1 (G1) strains circulating in mainland Portugal, suggesting an independent evolution of RHDV in Azores. More recently, the new variant RHDV (RHDVb) was detected in Azores. In mainland Portugal, current circulating strains resulted from recombination events between RHDVb and non-pathogenic or pathogenic G1 strains. To characterize the RHDVb strains from Azores, a ~ 2.5 kb fragment of the RHDV genome (nucleotide positions 4873–7323), including the complete sequence of the capsid gene VP60 (nucleotide positions 5305–7044), was amplified and sequenced. Samples were obtained from rabbits found dead in the field between December 2014 and March 2015 in the Azorean islands Flores, Graciosa, São Jorge, Terceira, Faial, Pico, São Miguel and Santa Maria. For VP60, the highest homology was found with Iberian RHDVb strains, while the upstream fragment revealed high similarity (~ 95 %) with Iberian G1 strains. Phylogenetic reconstruction based either on VP60 or VP10 grouped the Azorean strains with Iberian RHDVb strains. For the fragment upstream of VP60, the Azorean strains grouped with G1. Our results show that the RHDVb strains circulating in Azores are G1/RHDVb recombinants and we hypothesize that such strains had their origin in Iberian strains. The geographic isolation of Azores suggests that arrival of RHDVb was man-mediated. A network analysis further allowed us to trace virus dispersion in Azores: from an initial outbreak in Graciosa, RHDVb spread to São Jorge and Faial, to Terceira, Flores and Santa Maria, and finally to Pico; dispersion to São Miguel occurred later from Terceira. As the consequences of the presence of G1/RHDVb

strains in Azores are unpredictable, we suggest a continued monitoring and characterization of RHD outbreaks.

2. Introduction

In the Iberian Peninsula, two refuges originated two European rabbit subspecies: *Oryctolagus cuniculus algirus* and *Oryctolagus cuniculus cuniculus* [1]. The subspecies *O. c. cuniculus* is present throughout Europe, Australia and South America and originated all rabbit domestic breeds [1]. The subspecies *O. c. algirus* is restricted to the southwestern part of the Iberian Peninsula, and the islands of Azores, Madeira and Canary [1]. In the last 60 years, the Iberian wild rabbit populations suffered a great decline, mainly due to two viral diseases, myxomatosis and rabbit hemorrhagic disease (RHD) [2]. RHD is caused by a calicivirus, the rabbit hemorrhagic disease virus (RHDV), that was first reported in China in 1984 and rapidly spread worldwide, reaching the Iberian Peninsula in 1988 [reviewed in 3].

The study of the genetic diversity of RHDV allowed the distinction of six genogroups, G1–G6 [4]. Until 2011, only G1 strains were recovered from Iberian wild rabbit populations [5-7]; G6 (RHDVa) has also been detected, sporadically, in domestic rabbits [8,9]. This epidemiological pattern contrasts with that from other European countries where G1 was successively replaced by strains from other genogroups [4]. Recently, a new variant of RHDV, named RHDV2 or RHDVb, was described in France [10]. This new variant has unique features and dispersed quickly throughout Europe and now circulates in Spain, Portugal, Italy and United Kingdom [11-15]. In the Iberian Peninsula, RHDVb reduced rabbit numbers in ~ 80 % [16]. Characterization of full genomic sequences of Iberian RHDVb strains showed that these strains differ ~ 15 % in the capsid and ~ 22 % outside of the capsid from the other pathogenic and non-pathogenic strains [17]. Lopes *et al.* [18] further showed that Iberian RHDVb strains resulted from multiple recombination events involving non-pathogenic strains and pathogenic G1 strains. Indeed, while for the gene encoding the major capsid protein, VP60, and for the gene of the minor structural protein, VP10, all RHDVb viruses clustered together and apart from all the other strains, for the region encoding the non-structural proteins these strains appeared in three different clusters. Further analysis supported the existence of a single recombination breakpoint located in the 5’ region of VP60 compatible with the recombination pattern described.

The Portuguese Azores archipelago is located in the middle of the North Atlantic Ocean and is composed by nine islands (Fig. 2.8). Graciosa, São Jorge, Terceira, Faial and Pico constitute the central group; Flores, along with Corvo, compose the occidental group; São Miguel and Santa Maria belong to the oriental group. The European rabbit was introduced in Azores more than 500 years ago by the Portuguese settlers. Although the presence of RHD has been detected in the late 1980s, only in 2014 the circulating strains were characterized [9,19]. These strains were related to G3–G5 strains, but had unique features and differed at least 8 % from other RHDV strains and ~ 15 % from RHDVb, suggesting an independent evolution of RHDV in the Azorean islands after an initial introduction more than 17 years ago [19]. The presence of RHDVb was recently reported in the Azorean islands Graciosa, Terceira, São Jorge and Flores [20]. Since this report was based on a short fragment of the capsid gene (347 nucleotides, nt) and recombination clearly plays a role in shaping RHDVb diversity [18], we sequenced and analyzed a 2451 nt fragment, including the complete capsid gene, of RHDV strains recovered from rabbits found dead in Flores, Graciosa, Terceira, São Jorge, Faial, Pico, São Miguel and Santa Maria between December 2014 and March 2015.

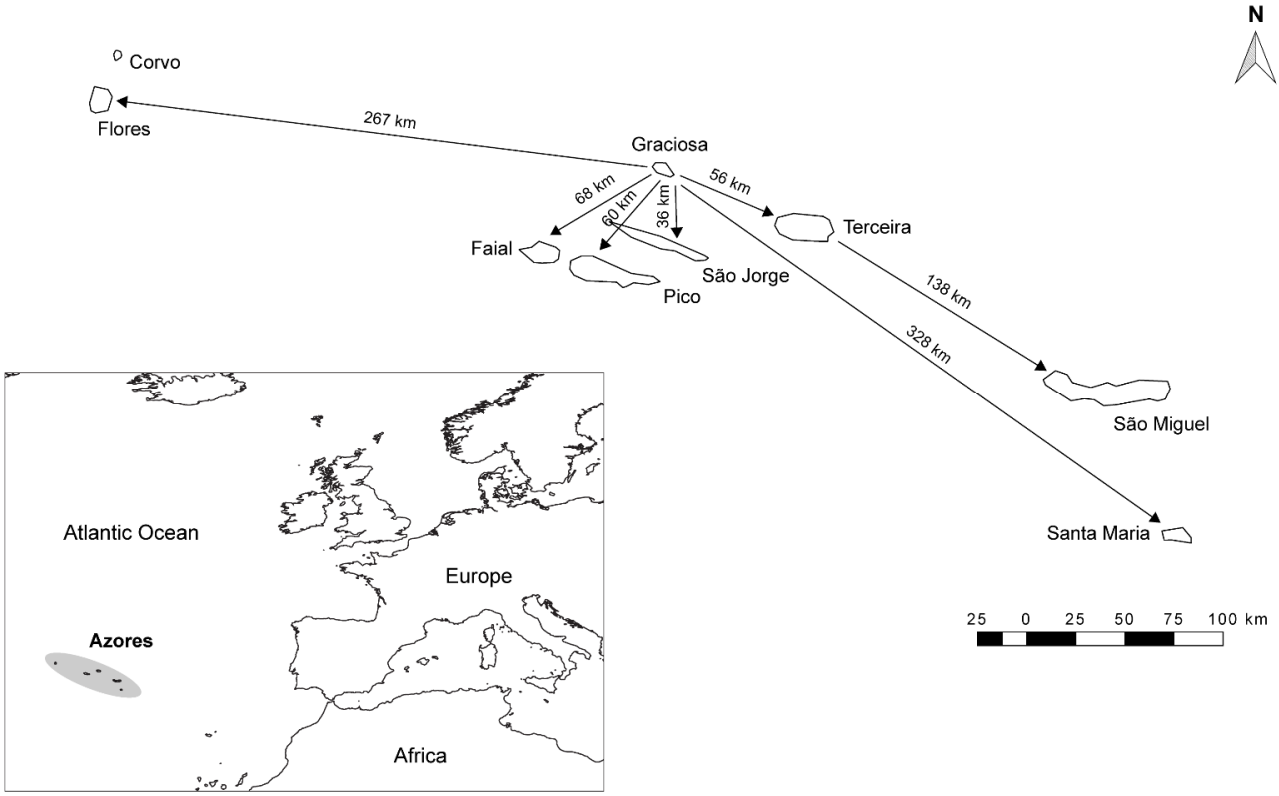

Figure 2.8 Map of the Portuguese Azores archipelago. Location of the islands is shown on the insert on the left (shaded light grey). Distances between Graciosa, where the first outbreak was detected, and Flores, Terceira, São Jorge, Pico, Faial and Santa Maria are indicated as well as between Terceira and São Miguel. RHDVb presence was detected in all islands except Corvo, where no rabbits exist.

3. Methods

Amplification and sequencing

Liver samples of 80 rabbits found dead in the field in the islands Flores (*n* = 12), Graciosa (*n* = 30), São Jorge (*n* = 7), Terceira (*n* = 4), Faial (*n* = 1), Pico (*n* = 2), São Miguel (*n* = 14) and Santa Maria (*n* = 10) were collected between December 2014 and March 2015 (Table 2.3), by the Direção Regional dos Recursos Florestais, Azores, and sent to the laboratory of CIBIO, InBIO-UP. No live animals were shot, trapped or handled to obtain tissues, such that no Animal Ethics permit was required. In the majority of the islands, dead rabbits were found in several places and carcasses were collected at the beginning of the outbreak (Table 2.3). At necropsy, the animals presented gross histopathological alterations compatible with RHD. A portion of liver sample (~ 30 mg) was homogenized in a rotor–stator homogenizer (Mixer Mill MM400, Retsch) at 30 Hz for 7 min. Extraction of viral RNA was performed using the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. Reverse transcription was performed using oligo(dT) as primers and SuperScript™ III Reverse Transcriptase (Invitrogen) according to the provided protocol. PCRs were performed as described elsewhere [18]. Briefly, samples were first screened by PCR with a pair of primers specific for RHDVb; positive samples were further PCR-amplified to obtain a fragment upstream of the capsid gene, the complete capsid gene and VP10. PCR products were purified and sequenced on an automatic sequencer ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems, Foster City, CA, USA) with the amplification primers. The sequences were deposited in GenBank under the following accession numbers: KT000290–KT000343.

Phylogenetic and phylogeographic analysis

The BioEdit software version 7.0.9.0 [21] was used to align the sequences along with other publicly available sequences, including from RHDVb (accession numbers are shown in Fig. 2.9). The alignment was screened for recombination using the RDP software version 4.26 [22] with the following parameters: sequences were set to linear; Bonferroni correction; highest acceptable *p*-value 0.05; 100 permutations. To infer the phylogenetic relationships, a first phylogenetic tree was drawn by using the full capsid sequences (nucleotide positions 5305–7044). A phylogenetic tree was also inferred for the region upstream of the capsid (nucleotide positions 4872–5304) and for VP10 (nucleotide positions 7025–7323). These trees were estimated using the Maximum Likelihood (ML) method available in MEGA6 [23] under the best-fit nucleotide substitution

model determined by the same software. Node support was determined from 500 bootstrap replicates of the ML trees. Pairwise genetic distances, between group mean distances and within group mean genetic diversity were also calculated in MEGA6. In order to represent the geographical dispersion and provide an alternative view of the phylogenetic relationships between the sequences retrieved from Azores, a phylogenetic network was constructed with the Phylogenetic Network Software version 4.6.1.3 (Fluxus-Technology, www.fluxus-engineering.com), using the Median-joining algorithm [24].

Table 2.3 List of the samples collected in the different islands.

Island (area in km^2)	Date	Municipality	Parish	No. of samples	Strains
Graciosa (61)	01-12-2014	Sta Cruz da Graciosa	Luz	10	CBGraciosa14-5 to 14-14
	15-12-2014	Sta Cruz da Graciosa	São Mateus	5	CBGraciosa14-15 to 14-19
	15-12-2014	Sta Cruz da Graciosa	Luz	5	CBGraciosa14-20 to 14-24
	15-12-2014	Sta Cruz da Graciosa	Guadalupe	10	CBGraciosa14-25 to 14-34
Terceira (402)	02-01-2015	Angra do Heroísmo	Porto Judeu	2	CBTerceira15-1, 15-2
	02-01-2015	Angra do Heroísmo	Posto Santo	2	CBTerceira15-3, 15-4
Flores (142)	05-01-2015	Laje das Flores	Mosteiro	8	CBFlores15-1 to 15-8
		Sta Cruz das Flores	Santa Cruz	4	CBFlores15-9 to 15-12
São Jorge (238)	02-02-2015	Velas	Rosais	7	CBSJorge15-1 to 15-3, 15-5 to 15-7
	02-02-2015	Velas	Sto Amaro	1	CBSJorge15-4
Santa Maria (97)	06-02-2015	Vila do Porto	Sto Espírito	5	CBSMaria15-1 to 15-5
	09-02-2015	Vila do Porto	Sto Espírito	3	CBSMaria15-6 to 15-8
	10-02-2015	Vila do Porto	Sto Espírito	2	CBSMaria15-9, 15-10
São Miguel (747)	09-02-2015	Povoação	Furnas	8	CBSMiguel15-1 to 15-6, 15-13, 15-14
	09-02-2015	Nordeste	Lomba da Fazenda	6	CBSMiguel15-7 to 15-12
Faial (173)	04-03-2015	Horta	Capelo	1	CBFaial15-1
Pico (445)	07-03-2015	Lajes do Pico	Piedade	1	CBPico15-2
	08-03-2015	Lajes do Pico	Piedade	1	CBPico15-1

4. Results and discussion

A fragment of ~ 2.5 kb was amplified and sequenced for 12 strains from Flores, 11 strains from Graciosa, five strains from São Jorge, three strains from Terceira, one strain from Faial, two from Pico, 11 from São Miguel and 9 from Santa Maria. A total of 55 nucleotide changes were observed between these strains; of these, two were diagnostic of strains from Flores and three of strains from Santa Maria. Twelve non-synonymous substitutions were observed, with six located in the capsid gene. Two of these

substitutions correspond to codons previously detected as being under positive selection [25] and more than two different amino acids are found for these positions. Mean genetic distances between islands for the sequenced fragment ranged between ~ 0.009 % (São Jorge–Faial) and ~ 0.399 % (São Miguel–Santa Maria). The mean genetic diversity of each island was 0.000547 ± 0.000212 for Santa Maria; 0.002089 ± 0.000640 for São Miguel; 0.000889 ± 0.000283 for Flores; 0.001550 ± 0.000438 for Graciosa; 0.001223 ± 0.000504 for São Jorge; 0.001086 ± 0.000570 for Terceira and 0.000814 ± 0.000530 for Pico (this was not computed for Faial as only one sequence was available). Comparison of each island with G1, G2, G3–G5 and G6 strains showed > 6 % of divergence for the fragment upstream of VP60, being more closely related to G1; > 18 % for the capsid and > 12 % for partial VP10. Both for VP60 and VP10, the Azorean strains were closely related to RHDVb strains from the Iberian Peninsula (divergences ranged from 2 % to 5 %).

A phylogenetic tree was inferred for the capsid sequences, including publicly available RHDV sequences of G1–G6, as well as non-pathogenic (RCV_Italy_1996; France_06-11_2006; Ashington_1998) and weakly pathogenic (MRCV_USA_2001) strains. Strains previously identified as belonging to G1–G6 clustered together in a highly supported group (Fig. 2.9a). The sequenced Azorean strains appear in a single highly supported subgroup closely related to the group composed of RHDVb strains from Spain and mainland Portugal (shaded light grey; Fig. 2.9a). The Azorean strains seem to be more closely related to Iberian RHDVb strains. This is in agreement with the results from the genetic distances and supports their identification as RHDVb.

Following the observation of a recombination breakpoint in the 5’ region of the capsid gene in the recently characterized RHDVb recombinant strains [18], we have also reconstructed the phylogeny based on (i) the fragment sequenced upstream of the capsid gene VP60 and (ii) the minor structural protein gene VP10. For the fragment upstream of the capsid (Fig. 2.9b), the Azorean strains grouped with G1 strains (shaded light grey), including previously identified G1/RHDVb recombinant strains (CBAlgarve14-1_Portugal; CBAlgarve14-3_Portugal; CBAlgarve14-4_Portugal; CBMert14-1_Portugal_2014; CBMert14-2_Portugal_2014) [18]; none of the Azorean strains clustered with other RHDVb strains or with G2-G6 (Fig. 2.9b). For VP10, the phylogenetic tree inferred was similar to that obtained for VP60 with the Azorean strains clustering with high support with the RHDVb group (only Portuguese and Spanish sequences are available; data not shown). These results indicate that the Azorean strains are recombinants between G1 and RHDVb, as observed for the most recent RHDVb strains circulating in the Iberian Peninsula [18]. Consistent with this, RDP detected these

sequences as G1/RHDVb recombinants with strong statistical support (*p*-values << 0.001; data not shown) and a recombination breakpoint was identified in the region upstream of the capsid initiation codon.

(a)

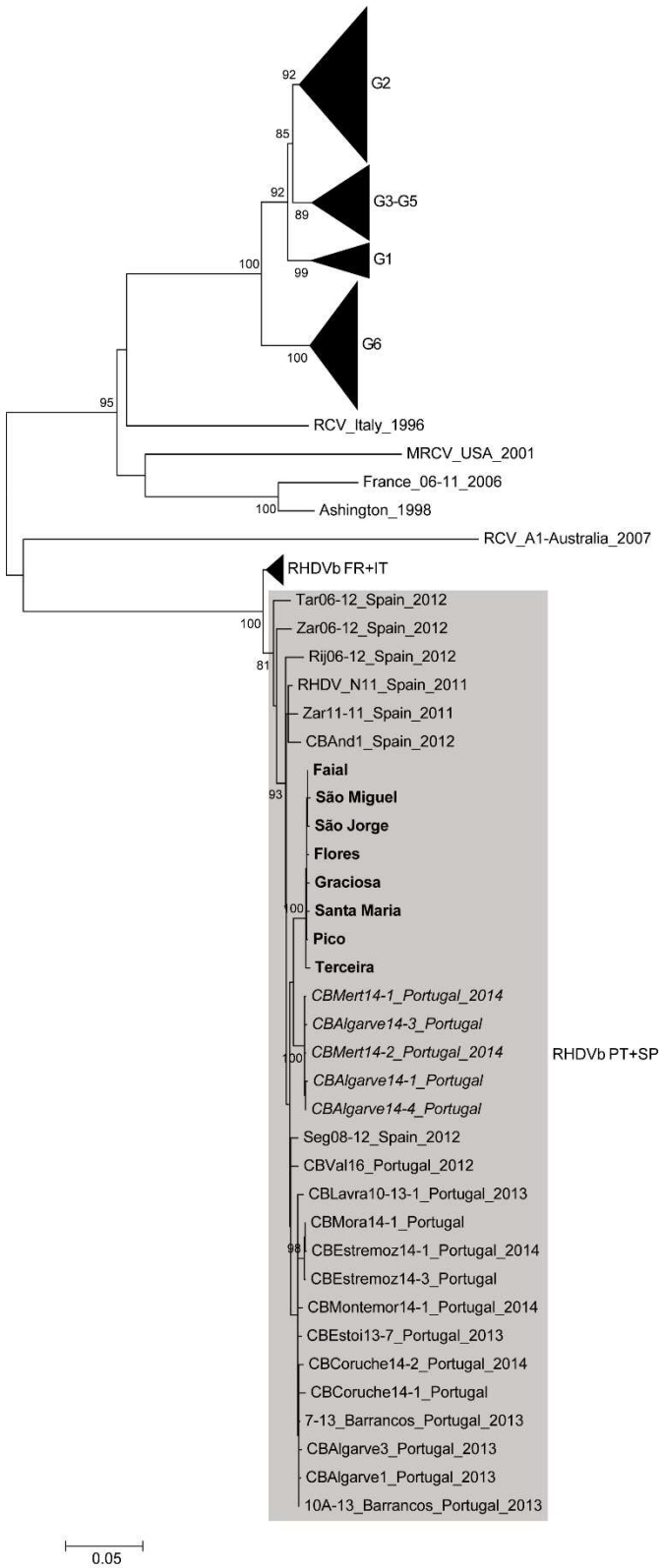

Figure 2.9 (cont.)

(b)

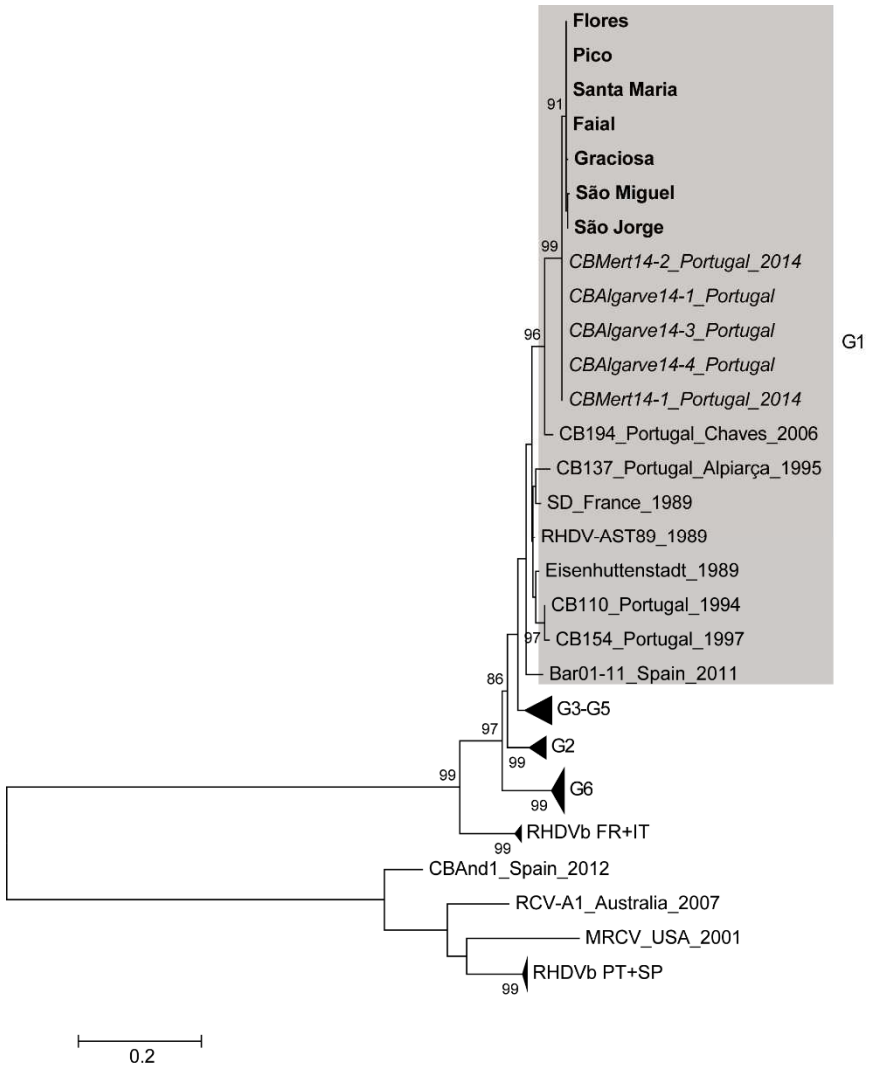

Figure 2.9 Maximum Likelihood (ML) trees of RHDV. (a) ML tree of the capsid gene and **(b)** ML tree of the region upstream of the capsid gene. The groups most closely related to the Azorean strains are shaded light grey; considering the low divergence between sequences, only one sequence per Azorean island was selected (in bold). Bootstrap values of the main nodes (≥ 75) are shown next to them. Scale bar indicates the nucleotide substitutions per site. To facilitate visualization, some groups were collapsed; GenBank accession numbers of the sequences used in this study are: KP090974-5, JF438967, EF558578, L48547, Z24757, Z29514, Z49271, KJ683894, KJ683903-4 (G1); AF231353, DQ189078, EU003579-80, EU650679-80, FJ212323, FR823355, GU373617-8, JN851735, M67473, RHU49726, RHU54983, KF594474-6, KJ606958-9, KF494906-52 (G2); AJ495856, AJ535092, AJ535094, AM085133, AY926883, AY928268-9, DQ189077, EF363035, EF558572, EF558574-5, EF558577, FN552800, FR823354, AY925210, X87607, Y15424, Y15427, KJ683893, KJ683900, KJ683906-8, KJ579156-60, KC595270, KC345614 (G3–G5); AB300693, AF258618, AJ302016, AJ303106, AJ969628, DQ069280-2, DQ205345, DQ841708, EF558581-4, EU003578, EU003581-2, EU250330, FJ212322, FJ794179-80, GU339228, GU564448, HE963222, HM623309, HQ917923, JF412629, JN165233-6, JN851729-34, JQ815391, JQ995154, KF270630, JX393309-12, KJ814617-22, KJ683902, KJ683905, KF537692-3 (G6); FR819781, HE819400, HE800529-32, FR819781, HE819400, HE800529-32 (RHDVb FR + IT); EU871528 (RCV-A1_Australia_2007); GQ166866 (MRCV_USA_2001); X96868 (RCV_Italy_1996); EF558587 (Ashington_1998); AM268419 (France_06-11_2006); KT000290 (Faial); KT000291 (Santa Maria); KT000306 (São Miguel); KT000313 (Flores); KT000329 (Graciosa); KT000337 (São Jorge); KT000339 (Terceira); KT000342 (Pico). KF442963-4, KM115714-6, KF442961-2, KM115697-8, KM115680-2, KM115711-3, KM115689, KM115683, KF442960, KM979445, KP090976, KM878681, KP129395-400 (RHDVb PT + SP; sequences identified in [18] as G1/RHDVb recombinants appear in italics).

In Azores, the presence of RHD was detected in the late 1980s, but the circulating strains were only recently characterized [9,19]. These studies showed that RHDV strains from Azores were more closely related to G3–G5 than to G1. Since divergence to G3–G5 strains was ~ 8 %, an independent evolution of RHDV in Azores was suggested following the initial introduction of an uncharacterized RHDV strain more than 17 years ago [19]. The Iberian strains were unlikely to be the source of those RHD outbreaks in Azores as, with the exception of the recent detection of RHDVb and some sporadic cases of G6 (RHDVa) in domestic rabbits, RHDV Iberian strains were typically G1 [6-9]. More recently, Duarte and co-workers reported the presence of RHDVb in the Azorean islands Flores, Terceira, Graciosa and São Jorge [20]. Our study confirms these results and extends the detection of RHDVb to the remaining Azorean islands Faial, Pico, Santa Maria and São Miguel (no rabbits exist in Corvo).

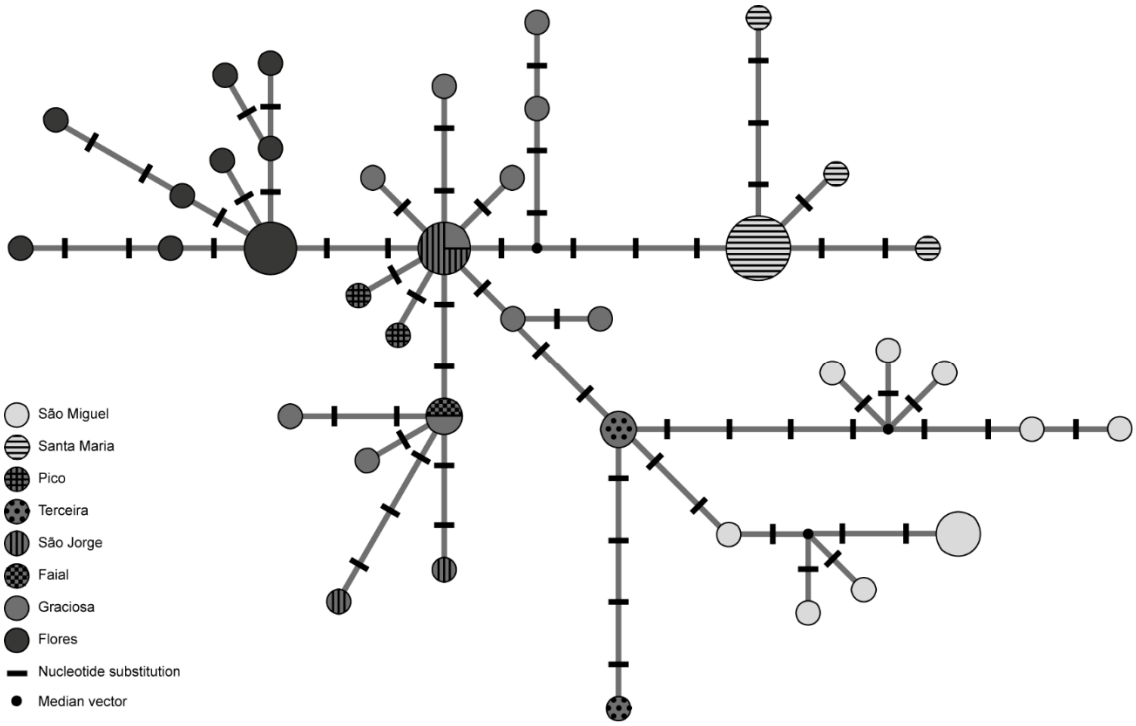

Figure 2.10 Median-joining network of RHDVb strains from Azores. Pie charts represent viral haplotypes (nucleotide positions 4873–7323), with colors/patterns indicating the island of sampling and the sizes reflecting the frequencies of the haplotypes. Median vectors, corresponding either to extinct or non-sampled sequences, are represented by black circles. The number of mutations separating each sequence is shown by a black line between each pair of haplotypes.

Since G1 strains no longer circulate in non-Iberian rabbit populations and G1/RHDVb strains are currently circulating in the Iberian Peninsula [18], it is highly plausible that the Azorean G1/RHDVb strains had their origin in the Iberian Peninsula.

Moreover, considering the geographic isolation of Azores, we hypothesize that arrival of RHDVb to Azores was man-mediated followed by a rapid virus spread. Our network analysis (Fig. 2.10) provides a picture into the virus dispersal route and epidemiology in the Azorean islands. An initial introduction seems to have occurred in Graciosa from where the virus rapidly expanded to other islands. According to official reports, the first dead rabbits in Azores were detected in Graciosa in late November 2014. Our results are in line with this: the highest haplotype diversity is found in Graciosa (Table 2.4) and a star-like topology is associated with the most ‘‘central’’ Graciosa haplotype consistent with a rapid viral expansion; in addition, viral sequences from Faial and São Jorge are identical to sequences from Graciosa supporting the colonization of those islands from Graciosa or with a strain identical to the strain circulating in Graciosa. Interestingly, while sequences from other islands appear in single clusters, sequences of São Jorge appear in different clusters, although always closely related to sequences from Graciosa. This might suggest multiple introductions from Graciosa to São Jorge. From Graciosa, the virus spread to Terceira, to Flores, to Santa Maria and finally to Pico (Table 2.3). Dispersion to São Miguel seems to have occurred from Terceira. Strikingly, the dispersal route does not always correlate with geographic proximity. While insects are considered important vectors for RHDV spread [26,27] and some of those vectors might be present in Azores (e.g. *Musca* spp., *Culex* spp., *Xenopsylla* spp. and *Spilopsyllus cuniculi* [28]), insect-mediated spread fails to explain the dispersion of RHDVb from Graciosa to Santa Maria, but not to São Miguel. Other vectors, such as birds [29-31], might account for RHDVb spread. In Azores, bird community is limited (currently 37 species and subspecies are considered regular breeders) and among island groups diversity decreases from east to west [32]. The common buzzard (*Buteo buteo rothschildi*) is the only resident raptor present in all Azorean islands and the yellow-legged Gull (*Larus michahellis*) was observed interacting with dead rabbits (personal observation). However, the role of these two species in the dispersion of RHDV in Azores is unknown and to our knowledge there are no regular movements of birds (migration) between islands; therefore, birds are also unlikely to have mediated RHDVb dispersion in Azores. Thus, considering the chronology of the outbreaks (Table 2.3), the distance between islands (Fig. 2.8) and the fact that arrival of RHDVb to Azores was man-mediated, it seems more likely that spread of RHDVb between islands was associated with anthropogenic movements.

Table 2.4 Frequencies of the haplotypes found on each island. Haplotypes shared between islands appear in bold.

Island	Haplotype (Frequency)
Faial	**1** (100 %)
Graciosa	**1**, 24, 25, 26, 27, 28, 29, 30, **31**, 32, 33 (0.91 %)
Santa Maria	2, 4, 5 (0.11 %), 3 (66.7 %)
São Miguel	6, 7, 8, 9, 11, 12, 13, 14 (0.91 %), 10 (27.3 %)
Flores	15, 16, 18, 19, 20, 21, 22, 23 (0.83 %), 17 (33.3 %)
São Jorge	34, 35 (20 %), **31** (60 %)
Terceira	36 (33.3 %), 37 (66.7 %)
Pico	38 (50 %), 39 (50 %)

5. Conclusions

In the Iberian Peninsula, RHDVb quickly replaced G1 as the main circulating strain, possibly reflecting a yet to be described selective advantage [33]. The presence of RHDVb in Iberian Peninsula is associated with a negative impact in the ecosystem [16] and in the economy. In Azores, and in an effort to contain RHD outbreaks, the hunting activity is now suspended (indefinitely) in all islands. Since the consequences of the presence of RHDVb in Azores are unpredictable, continued monitoring of the RHD outbreaks and characterization of the circulating strains in the Azorean islands is advisable. In addition, the presence of RHDVb in Azores provides a unique situation to study the dynamics and the evolution of this new variant, including its ability to recombine with strains from genogroups other than G1.

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Chapter 3

EBHSV: virus evolution and attachment factors

Paper V. Lopes AM, Gavier-Widén D, Le Gall-Reculé G, Esteves PJ, Abrantes J: **Complete coding sequences of European brown hare syndrome virus (EBHSV) strains isolated in 1982 in Sweden**. *Archives of Virology* 2013, **158**:2193-2196.

Paper VI. Lopes AM, Capucci L, Gavier-Widén D, Le Gall-Reculé G, Brocchi E, Barbieri I, Quéménér A, Le Pendu J, Geoghegan JL, Holmes EC, et al.: **Molecular evolution and antigenic variation of European brown hare syndrome virus (EBHSV)**. *Virology* 2014, **468-470**:104-112.

Paper VII. Lopes AM, Lora M, Le Moullac-Vaidye B, Ruvoën-Clouet N, Breiman A, Galanina OE, Bovin NV, Esteves PJ, Abrantes J, Le Pendu J: **A new attachment factor in the *Lagovirus* genus: the European brown hare syndrome virus (EBHSV) uses *N*-acetylglucosamine O-glycans to attach to host cells**. In preparation.

Complete coding sequences of European brown hare syndrome virus (EBHSV) strains isolated in 1982 in Sweden

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1. Abstract

European brown hare syndrome (EBHS) is characterized by high mortality of European brown hares (*Lepus europaeus*) and mountain hares (*Lepus timidus*). European brown hare syndrome virus (EBHSV) and the closely related rabbit hemorrhagic disease virus (RHDV) comprise the genus *Lagovirus*, family *Caliciviridae*. In contrast to RHDV, which is well studied, with more than 30 complete genome sequences available, the only complete genome sequence available for EBHSV was obtained from a strain isolated in 1989 in France. EBHS was originally diagnosed in Sweden in 1980. Here, we report the complete coding sequences of two EBHSV strains isolated from European brown hares that died with liver lesions characteristic of EBHS in Sweden in 1982. These sequences represent the oldest complete coding sequences of EBHSV isolated from the original area of virus diagnosis. The genomic organization is similar to that of the published French sequence. Comparison with this sequence revealed several nucleotide substitutions, corresponding to 6 % divergence. At the amino acid level, the Swedish strains are 2 % different from the French strain. Most amino acid substitutions were located within the major capsid protein VP60, but when considering the amino acid sequence length of each protein, VP10 is the protein with the highest percentage of amino acid differences. The same result was obtained when Swedish strains were compared. This evolutionary pattern has not been described previously for members of the genus *Lagovirus*.

2. Introduction

European brown hare syndrome (EBHS) is a highly contagious viral disease that affects wild and captive European brown hares (*Lepus europaeus*) and mountain hares (*Lepus timidus*). The disease was first diagnosed in Sweden in 1980 [1], but successful recovery of the causative virus was achieved from a sample collected earlier [2]. In addition, there are reports of outbreaks compatible with EBHS since 1976 in England [3], and antibodies against the virus have been detected in sera of hares archived since 1962 [4]. Moreover, Scandinavian hunters were aware of the disease in the early 1970s [5]. This suggests that despite being unnoticed, the virus was circulating in European hare populations, most likely as a less virulent form. Since then, the disease has been recognized in several other European countries [6-15] and, more recently, in South America [16].

The etiological agent of EBHS, European brown hare syndrome virus (EBHSV), is a calicivirus that, along with the closely related rabbit hemorrhagic disease virus (RHDV), composes the genus *Lagovirus* [17]. The genome of EBHSV is a single-stranded positive-sense RNA, ~ 7.4 kb in length that encompasses two narrowly overlapping open reading frames (ORFs). ORF1 (nucleotides 9-7010) codes for the non-structural proteins, including the replicative machinery, and the structural capsid protein VP60. ORF2 (nucleotides 7006-7350) codes for VP10, the function of which remains unclear [18].

Since the determination of the first complete genomic sequence of EBHSV from a strain isolated in France in 1989 (GenBank accession number Z69620) [18], molecular information on EBHSV has been limited and fragmented. Indeed, most studies, even the most recent, rely on partial sequences of the capsid protein [19]. Since the genome of lagoviruses is prone to recombination, which confounds the establishment of true phylogenetic relationships [20,21], determination of complete coding sequences is essential for understanding evolution of such viruses. Furthermore, this would allow a comparison between RHDV and EBHSV genetic diversity. Here, we have determined the complete coding sequences of two EBHSV strains isolated from liver tissue of hares collected in 1982 in Sweden. The sequences obtained correspond to the oldest complete coding sequences of EBHSV isolated in the original area of diagnosis of the virus.

3. Methods

The samples consisted of livers from dead hares collected in Höör, Skåne County, Sweden, on November 11, 1982 by the National Veterinary Institute (SVA), Uppsala, Sweden, and were stored at -20 ºC. At necropsy, the hares presented with histological liver lesions characteristic of EBHS-infected animals, which consisted of coagulative periportal to massive hepatocellular necrosis [22].

For RNA extraction, 30 mg of each liver tissue was used. The tissue was homogenized using a rotor-stator homogenizer (Mixer Mill MM400, Retsch) at 30 Hz for 7 min. Total RNA was extracted using an RNeasy Mini Kit (QIAGEN) according to the manufacturer's instructions. Viral RNAs were reverse transcribed using oligo(dT) as primers (Invitrogen) and SuperScript™ III Reverse Transcriptase (Invitrogen).

For the initial screening by PCR, the primer pair U38 (5' CAGCGGGCACTGCTACCACAGCATC 3') and EBHS9 (5' CCAGCCCAACCAGCRTACAT 3') was used. These primers amplified a fragment of 307 base pairs (bp) of the gene encoding the capsid protein VP60. Complete genomic sequences were then obtained with eight pairs of primers (Table 3.1). Amplification by PCR was performed with 0.6 µL of the cDNA reaction in a final volume of 10 µL containing 5 µL of Phusion Flash High-Fidelity PCR Master Mix (Thermo Scientific) and 2 pmol of each oligonucleotide. Cycle conditions were 3 min at 98 ºC, followed by 40 cycles of 30 s at 98 ºC, 30 s at the annealing temperature and extension at 72 ºC (see Table 3.1 for the annealing temperatures and extension times). Final extension was carried out for 5 min at 72 ºC. After purification, PCR products were sequenced on an automatic sequencer (ABI PRISM 310 Genetic Analyzer, PE Applied Biosystems). The complete coding sequences of the two strains were deposited in GenBank under accession numbers KC832839 and KC832838 for O4021-9_1982 and O4022-10_1982, respectively.

4. Results and discussion

A total of 7342 nucleotides were sequenced for each strain, corresponding to the complete ORF1 and ORF2. Analysis of the genomic organization revealed a structure similar to that of the complete published sequence of EBHSV [18]. ORF1 codes for a polyprotein of 2334 amino acids that include the non-structural proteins (5'-p16, p23/p26, 2C-like helicase, p29, VPg, 3C-like protease and RNA-dependent RNA polymerase

[RdRp]-3') and the major capsid protein (VP60; 3' of the polyprotein). ORF2 codes for VP10, a protein of 114 amino acids.

Table 3.1 List of the primers used for PCR amplification and sequencing. Ta: annealing temperature.

Primer	Primer sequence (5'-3')	Position*	Ta (ºC)	Extension time
EBHSV0001F	GTGAAATTATGGCGGTTGCG	1-20	54	1 min 15 s
EBHSV1085R	GGACTGAGTCCTCAAACTTG	1085-1104		
EBHSV0908F	CCTGCTGGATTGGACAAATGAC	908-929	52	1 min 15 s
EBHSV1977R	GAAGGTGAGGTGAGACATGTC	1977-1997		
EBHSV1761F	CCTCTCAACTGTGACAAGGTTG	1761-1782	49	1 min 15 s
EBHSV2745R	GGTCACCAGAGTATCTCACAAG	2745-2766		
EBHSV2615F	CAATCCGGTGTGTGCATATG	2615-2634	49	1 min 15 s
EBHSV3835R	CCTGGAACCTTTGCATACCTTG	3835-3856		
EBHSV3767F	CATTGACTACCGTGGACTTG	3767-3786	51	1 min 15 s
EBHSV4604R	GAACGGTCATTGGAAGTGAC	4604-4623		
EBHSV4503F	GATTCCACGATGTCACCATG	4503-4522	53	1 min 15 s
EBHSV5320R	AGGAACAGATGCTGTGGTAG	5320-5339		
EBHSV_VP60_0001F	ATGGAGGGTAAGCCWCGGGCTGA	5283-5305	53	1 min 30 s
EBHSV_VP60_1728R	GACATAGGAATATCCAGTGGTGGC	6987-7010		
EBHSV6911F	GACAGACCTCATTGACGTG	6911-6929	54	45 s
EBHSV7337R	CAAAYCGCTAGGCGTTACTC	7337-7356		

*According to the published EBHSV sequence (GenBank accession number Z69620)

At the nucleotide level, comparison of the ORF1 sequences of the two isolates showed 168 differences that translated into 17 amino acid differences (0.7 %). No amino acid differences were observed between the Swedish sequences in RdRp, and most of the differences (5) were located in VP60. Interestingly, when considering the amino acid length of each protein, p16 had differences in ~ 2 % of the amino acids, p23/p26 and p29 in ~ 1 %, VPg and VP60 in ~ 0.9 %, 3C-like protease in ~ 0.7 % and 2C-like helicase in ~ 0.3 %. In ORF2, the two sequences differed by 11 nucleotides that corresponded to 3 non-synonymous replacements, i.e., differences in ~ 3 % of the amino acids, if considering amino acid length.

On average, classical RHDV isolates exhibit a maximum of 10 % nucleotide differences and 6 % amino acid differences [23]. Here, the comparison of the Swedish strains and the published French strain, which were collected 7 years apart showed 6 % of divergence at the nucleotide level and 2 % at the amino acid level. For ORF1, 490 variable sites were observed, but multiple substitutions were detected at some of these sites, and thus, the total number of mutations was 494. This corresponded to 431 synonymous substitutions and 63 non-synonymous replacements, mostly located in

VP60 (28 % of all non-synonymous substitutions). For ORF2, 23 polymorphic sites were detected, with a total number of 17 silent changes and 6 non-synonymous replacements. When considering amino acid length for each protein, ~ 5 % of the amino acid differences are found in VP10, ~ 4 % in p16 and p23/p26, ~ 3 % in 2C-like helicase, p29, VPg and VP60, and ~ 1 % in 3C-like protease and RdRp.

Regarding the ability of the EBHSV 3C-like protease to cleave the ORF1-encoded polyprotein to give rise to the mature viral proteins, no experimental data exist. Nevertheless, a previous comparison with RHDV indicated that at least four cleavage sites are conserved (2C-like helicase/ p29; VPg/3C-like protease; 3C-like protease/RdRp and RdRP/VP60) [18]. For the Swedish strains, three of these sites are conserved, but the cleavage site ET between the 3C-like protease and RdRp is replaced by EN. This has also been reported for the French EBHSV strain, and cleavage efficiency is thought not to be affected [18,24]. The other cleavage sites that were identified for RHDV, p16/p23 and p29/VPg, are conserved [25], but variation was observed between the Swedish strains at the p23/2C-like helicase boundary (E<u>D</u>/E<u>E</u>; ED for RHDV). The implications of such substitutions for cleavage efficiency are unclear.

5. Conclusions

The genomic organization of the sequenced EBHSV strains is similar to that of the reported French sequence, and most of the cleavage sites are conserved. As expected, most amino acid differences were observed in the VP60 protein. However, when considering protein size, VP10 is the protein with the most amino acid differences, a pattern that has not been reported for lagoviruses. The sequences described correspond to the oldest sequences of EBHSV and should contribute to a better understanding of the evolution of EBHSV. In addition, the results obtained reinforce the importance of obtaining complete genomic sequences for other members of the genus *Lagovirus*.

6. References

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Molecular evolution and antigenic variation of European brown hare syndrome virus (EBHSV)

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1. Abstract

European brown hare syndrome virus (EBHSV) is the etiological agent of European brown hare syndrome (EBHS), a disease affecting *Lepus europaeus* and *Lepus timidus* first diagnosed in Sweden in 1980. To characterize EBHSV evolution we studied hare samples collected in Sweden between 1982 and 2008. Our molecular clock dating is compatible with EBHSV emergence in the 1970s. Phylogenetic analysis revealed two lineages: Group A persisted until 1989 when it apparently suffered extinction; Group B emerged in the mid-1980s and contains the most recent strains. Antigenic differences exist between groups, with loss of reactivity of some MAbs over time, which are associated with amino acid substitutions in recognized epitopes. A role for immune selection is also supported by the presence of positively selected codons in exposed regions of the capsid. Hence, EBHSV evolution is characterized by replacement of Group A by Group B viruses, suggesting that the latter possess a selective advantage.

2. Introduction

European brown hare syndrome (EBHS) is a contagious disease characterized by severe necrotizing hepatitis in European hares (*Lepus europaeus*) and, to a lesser extent, in mountain hares (*Lepus timidus*) [1]. In the acute form hares die within a few hours after the appearance of clinical signs [1]. Although infected, young hares do not develop EBHS, possibly due to natural resistance [2-4]. The etiological agent of EBHS is the European brown hare syndrome virus (EBHSV), a non-enveloped, single-stranded, positive-sense RNA virus. EBHSV belongs to the genus *Lagovirus*, family *Caliciviridae* [5]. The other member of this genus, rabbit hemorrhagic disease virus

(RHDV), is responsible for rabbit hemorrhagic disease (RHD), an acute lethal hepatitis first described in China in 1984 that affects wild and domestic adult European rabbits (*Oryctolagus cuniculus*) [6]. RHD and EBHS share similar clinical signs and histopathology, as well as a similar epidemiology [reviewed in 7]. Despite the close phylogenetic relationship between EBHSV and RHDV, their antigenic profiles are distinct [2,8,9]. They also differ in their evolutionary patterns with a strongest geographical structure for EBHSV [10]. EBHS was first reported in Sweden in 1980 [1] and in the following years in several other European countries [8,11-15]. Indeed, phylogenetic studies suggested that virus dispersion occurred from Sweden to west, east and southern Europe [13]. However, EBHSV likely emerged in the early 1970s, as suggested by descriptions of hares with lesions consistent with the disease in 1976 in England [16]. Antibodies against the virus have also been found in archived sera [17], and the virus was detected by PCR in samples collected in Sweden before 1980 [18], further supporting an earlier emergence.

To understand the phylodynamics of EBHSV since its initial description in 1980 in Sweden, we analyzed hare samples collected between 1982 and 2008 in several Swedish geographic regions, as they reflect the spread of the virus in a naïve environment, along with samples from countries where the disease was later identified. In addition, the antigenic profiles of EBHSV strains were assessed with a panel of monoclonal antibodies (MAbs) and their association with patterns of virus evolution was determined.

3. Methods

Virus samples, RNA extraction and cDNA synthesis

Liver samples from 95 *Lepus europaeus* and 9 *Lepus timidus* found dead in the field were collected in different regions of Sweden between 1982 and 2008 (Fig. 3.1), resulting from passive surveillance. Each frozen liver was thawed and either 100 µL of the obtained exudates or up to 30 mg of the liver were collected. When using liver pieces, the tissue was homogenized in a rotor-stator homogenizer (Mixer Mill MM400 from Retsch) at 30 Hz for 7 min. Total RNA was extracted using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Reverse transcription was performed using oligo(dT) as primers (Invitrogen) and SuperScript™ III Reverse Transcriptase (Invitrogen) according to manufacturer's instructions. Sixteen *Lepus europaeus* liver samples collected throughout France between 2001 and 2008 by the SAGIR network

(French Wildlife Health Surveillance Network) [19] were also analyzed. The presence of EBHSV in these samples was confirmed by ELISA in a laboratory of veterinary analyses (Anjou Laboratoire, Angers, France). The five samples collected before 2003 were previously partially sequenced and genotyped [10]. For the other samples, RNA extraction and cDNA synthesis were performed as described above except that SuperScript™ II Reverse Transcriptase (Invitrogen) was used for the reverse transcription.

Amplification and sequencing

The complete sequence of the gene encoding the capsid protein was amplified using the primers EBHSV_VP60_0001F+EBHSV_VP60_0885R and EBHSV_VP60_0813F+EBHSV_VP60_1728R for the Swedish samples and EBHSV-29F+EBHSV-1795R for the French samples (Table 3.2). After purification, PCR products were sequenced on an automatic sequencer ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems). The Swedish and French sequences were determined using the PCR primers as well as several primers designed in the inner region of the DNA template (primer sequences available upon request). These sequences have been deposited in GenBank and assigned the following accession numbers: KJ679513-KJ679566; AJ971301; AJ971304-6; AJ971311; AJ971315; FN689419-21; AM408588; AM887765; AM933648-50; HF571039-40. Samples fully sequenced for the VP60 gene were aligned using BioEdit version 7.0.9.0 [20]. Four reference EBHSV public sequences that were collected in 1989 and 1992 in France, Italy and Germany (GenBank accession numbers Z69620, X98002, KJ923230 and U09199, respectively) were also included in the study. In total, 74 complete VP60 sequences were available for evolutionary analysis.

Analysis of recombination events

Prior to phylogenetic analysis, the sequence alignment was screened for recombination using the RDP, Geneconv and Bootscan methods implemented in the Recombination Detection Program (RDP) version 3.44 [21] with the following parameters: sequences were set to linear, Bonferroni correction, highest acceptable *p*-value=0.05, 100 permutations. Only recombination events detected by all three methods ($p < 0.05$) were considered as significant.

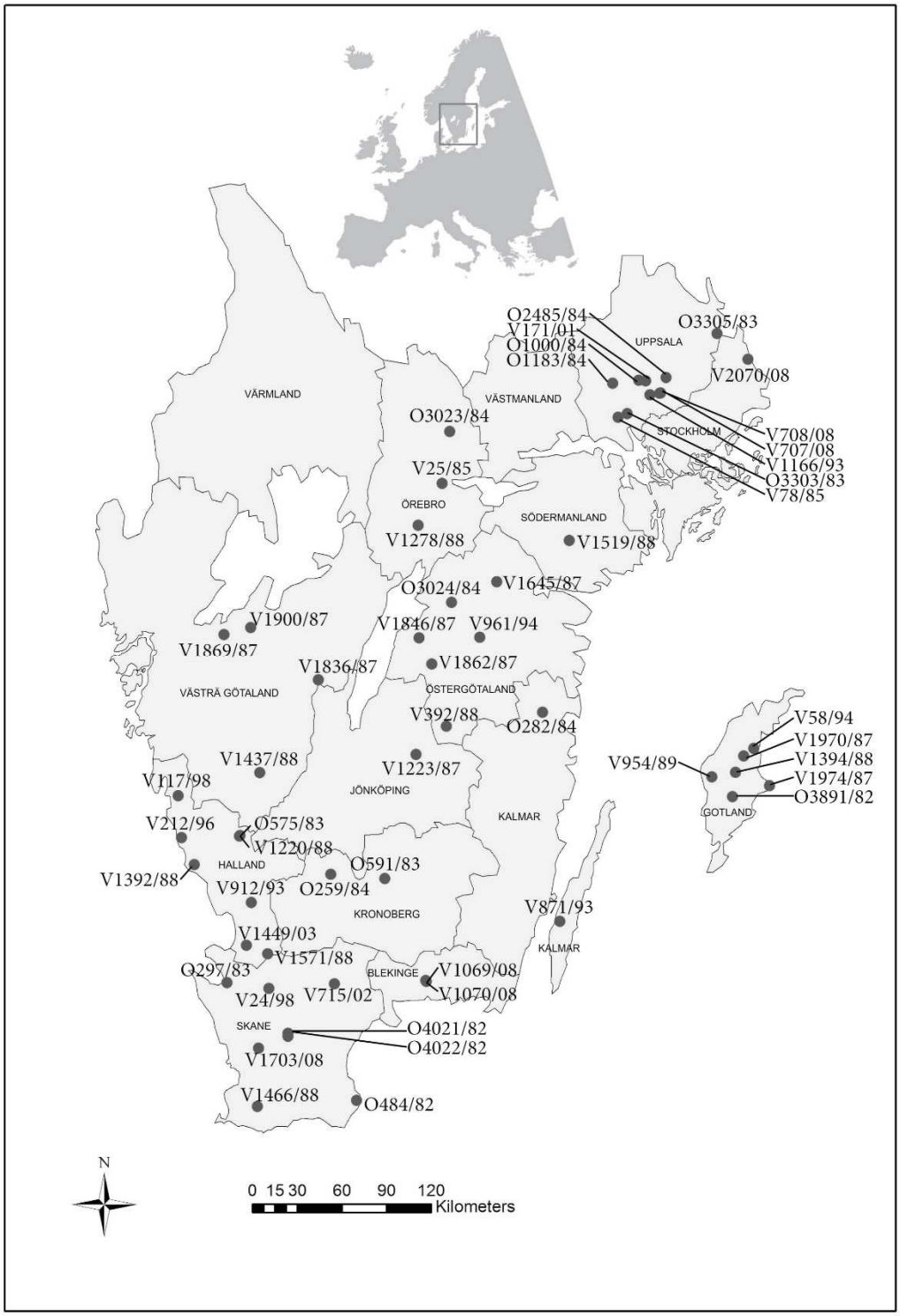

Figure 3.1 Map of Southern Sweden. Location of the EBHSV positive sequenced samples is shown and counties are indicated.

Phylogenetic and phylogeographic analysis

The phylogenetic relationships among the capsid sequences were first estimated using the Maximum Likelihood (ML) method available within the PhyML package [22].

This analysis utilized the GTR+Γ model of nucleotide substitution and a combination of SPR and NNI branch-swapping, along with a bootstrap resampling analysis (1000 replications).

Table 3.2 Sequence and nucleotide position of the PCR and sequencing primers.

Primer	Primer sequence (5'-3')	Position*
EBHSV_VP60_0001F	ATGGAGGGTAAGCCWCGGGCTGA	5283-5305
EBHSV_VP60_0813F	CAGRCACTGGAAYATGAATGG	6095-6115
EBHSV_VP60_0885R	CTTGGATGRTCAATGTCGTC	6147-6166
EBHSV_VP60_1728R	GACATAGGAATATCCAGTGGTGGC	6987-7010
EBHSV-29F	CGGACGATAGTTTTGTGAATGT	5260-5281
EBHSV-1795R	TAGAAGCTCCAGCCAATGTT	7026-7045

* Position relative to GenBank accession number Z69620.

A second phylogenetic analysis was then performed using the Bayesian Markov chain Monte Carlo (MCMC) method available in the BEAST package [version 1.7.5; 23], which also allowed us to estimate the rate of nucleotide substitution and time to the Most Recent Common Ancestor (tMRCA) of our EBHSV sequences. The GTR+Γ+I (i.e. general time reversible substitution matrix; Γ, distribution of among-site rate variation, and I, proportion of invariant sites) substitution model and a variety of coalescent (constant population size, Bayesian skyline, Bayesian skyride) models were used with relatively uninformative priors, all of which produced highly comparable results (see Results). To allow for rate variation among lineages, an uncorrelated lognormal relaxed molecular clock was used in all cases. Posterior probabilities were determined after 100 million generations, after which parameters had converged. Statistical uncertainty was reflected in values of the 95 % highest posterior density (HPD). Finally, a Maximum Clade Credibility (MCC) tree was summarized in TreeAnnotator using the posterior distribution of trees and excluding a 10 % burn-in.

To reveal general aspects of phylogeography, EBHSV sequences were classified to either their country of origin (France, Germany, Italy) or their county (*n*=13) of origin within Sweden (Fig. 3.1), producing a total of 16 different geographic states. To determine whether there was more EBHSV clustering by place of sampling than expected by chance alone, we utilized two phylogeny-trait association tests (Association Index – AI, Parsimony Score – PS) available in the BaTS package [24]. In addition, the Maximum Clade (MC) statistic was used to determine which individual countries/counties showed the strongest spatial clustering. Phylogenetic uncertainty in the data was

incorporated through the use of the posterior distribution of trees determined from the BEAST analysis above, with 1000 random permutations of sampling locations undertaken to create a null distribution for each statistic.

Analysis of selection pressures

Selection pressures acting on EBHSV capsid sequences were assessed as the ratio of non-synonymous (d_N) to synonymous (d_S) nucleotide substitutions per site (i.e. d_N/d_S). A variety of methods available in the HyPhy package were run on the Datamonkey web interface [25,26]: single likelihood ancestor counting (SLAC), fixed effects likelihood (FEL), random effects likelihood (REL), mixed effects model of evolution (MEME) and the fast unbiased Bayesian approximation (FUBAR). Only sites detected by at least two methods with statistically significant values were considered as being subject to positive selection under these criteria (p-value < 0.05 for SLAC, FEL and MEME; posterior probability > 0.95 for REL and FUBAR). The Branch Site-REL method was also employed to detect selection on individual branches of the EBHSV tree [27].

Antigenic analysis of EBHSV strains

The antigenic profile of nine PCR-positive strains was determined using a panel of specific monoclonal antibodies (MAbs) produced against Bs91/Italy/1991 strain [28]. Four to five samples from each main branch of the EBHSV phylogeny were selected. The antigenic profile of the strains was obtained using a sandwich ELISA as previously described [29] with a hare anti-EBHSV immune serum adsorbed to the plate and MAbs at a concentration of approximately 100 ng/mL. For each MAb, the absorbance values were transformed in percentage of reactivity using as 100 % of reactivity the average OD values of all the samples with an OD value equal to the strain used as standard (Bs91/Italy/1991; ± 0.2 OD).

MAbs epitope prediction

Nucleotide sequences of the nine strains used in the antigenic analyses, as well as that of the strain Ita92/Italy/1992, were aligned and translated. The epitope of each MAb was inferred comparing the variable amino acid positions between reactive and

non-reactive strains. Intermediate values of MAb reactivity were not considered as they might result from other interfering factors. Indeed, the results were obtained from livers of dead hares collected in the field and stored frozen for several years, which might have caused a partial virus degradation. Nevertheless, most of the older samples used were fully reactive for all the MAbs tested, indicating an intact viral capsid. Inferred epitopes were then mapped on the VP60 structural model (see below).

Homology modelling of EBHSV strains

Secondary structure prediction was performed with Jpred (http://www.compbio.dundee.ac.uk/www-jpred), Prof (http://www.aber.ac.uk/phiwww/prof/) and Psi-pred (http://bioinf.cs.ucl.ac.uk/psipred). Structure templates were selected according to e-value criterion using the WU-BLAST tool (http://www.ebi.ac.uk/Tools/sss/wublast/). X-ray crystallographic structures with a resolution less than 2.1 Å were arbitrarily retained. Sequence alignment of selected templates and the sequence of the capsid protein of EBHSV strain O282/Sweden/1984 was first calculated from a pairwise structure alignment automatically generated by the Align123 algorithm implemented under Discovery Studio 4.0 (DS4.0, Accelrys, San Diego), and then manually optimized (see Supplementary Fig. 1).

Homology modelling was performed with the Modeler program under DS4.0 using X-ray crystallographic structures of domain S and P of the major capsid protein from RHDV (PDB codes: 4EJR and 4EGT, respectively). The generated models were checked using Verify3D (http://nihserver.mbi.ucla.edu/Verify3D), ProsaII (http://prosa.services.came.sbg.ac.at/prosa.php) and Pro-check (http://www.ebi.ac.uk/thornton-srv/databases/pdbsum/Generate.html) programs, and the structures with the best stereo-chemical and folding qualities were retained. A series of energy minimizations was carried out with CHARMm force field implemented under DS4.0 in a three-step procedure to relax progressively the structures (1: all heavy atoms fixed; 2: heavy atoms of the backbone fixed; 3: heavy atoms of the secondary structure backbone fixed) using 200 steepest descent steps with convergence obtained at 0.1 rmsg, followed by conjugate gradient steps with convergence obtained at 0.001 rmsg. The refined structures of both the S and P domains were then superimposed into the atomic model of RHDV (PDB code 3J1P) to correctly position the two domains relative to each other. Capsid monomers are divided into a buried shell (S) domain connected to the protruding (P) domain that is exposed on the surface; the P domain is subdivided into P1 (stem of arc) and P2 (top of arc) [reviewed in 7]. The other strain models were

then obtained from the minimized model structure of O282/Sweden/1984 using the “Build Mutant” tool of DS4.0 with secondary structure restraints and a distance cut-off of 5 Å centered on the mutated residues for including neighboring residues in optimization (Supplementary Fig. 2).

4. Results

Seventy-five Swedish samples were positive for EBHSV by PCR (72 % of the original dataset) and, of those, 54 were successfully sequenced for the complete capsid VP60 gene. The final dataset included EBHSV strains from 52 *Lepus europaeus* and from two *Lepus timidus* specimens. Additionally, 16 French samples were also amplified and sequenced for the complete VP60 gene.

Both the Maximum Likelihood (ML) and Bayesian (MCC) trees revealed the presence of two main phylogenetically distinct groups (Group A and Group B), with no evidence for recombination. The MCC tree (GTR+Γ+I substitution model, constant population size coalescent prior) is shown in Fig. 3.2, while the ML tree is available from the authors on request. Group A comprises Swedish strains from 1982 to 1989, thus representing the early evolutionary history of EBHSV. In contrast, Group B contains two Swedish strains from 1988, all those from the 1990s and 2000s, as well as strains sampled from other European countries (France, Germany and Italy). The strong time-dependence (i.e. clock-like evolution) in the data, such that older strains occupy a more basal phylogenetic position than those sampled more recently, was confirmed in a regression of root-to-tip genetic distances against year of sampling inferred from the ML tree (correlation coefficient = 0.96).

Our Bayesian MCMC analysis revealed that the mean rate of evolutionary change in EBHSV was approximately 3×10^{-3} nucleotide substitutions per site per year (subs/site/year), with little variation across coalescent models (range of 95 % HPD values of $2.6–3.6 \times 10^{-3}$ subs/site/year across all models). Under this evolutionary rate, the tMRCA for EBHSV was estimated to be between 28 and 39 years (range of 95 % HPD values), which accords to the years 1969–1980. Additionally, our phylogeographic analysis revealed a significant clustering of sequences by country/province of origin under both the AI and PS phylogeny-trait association statistics ($p < 0.01$), such that there is restricted viral traffic across this spatial region. Similarly, the MC statistic revealed significant clustering in France and in six of the 13 Swedish counties (Blekinge, Gotland,

Örebro, Östergotland, Uppsala and Västra Götaland) that also had the largest sample sizes.

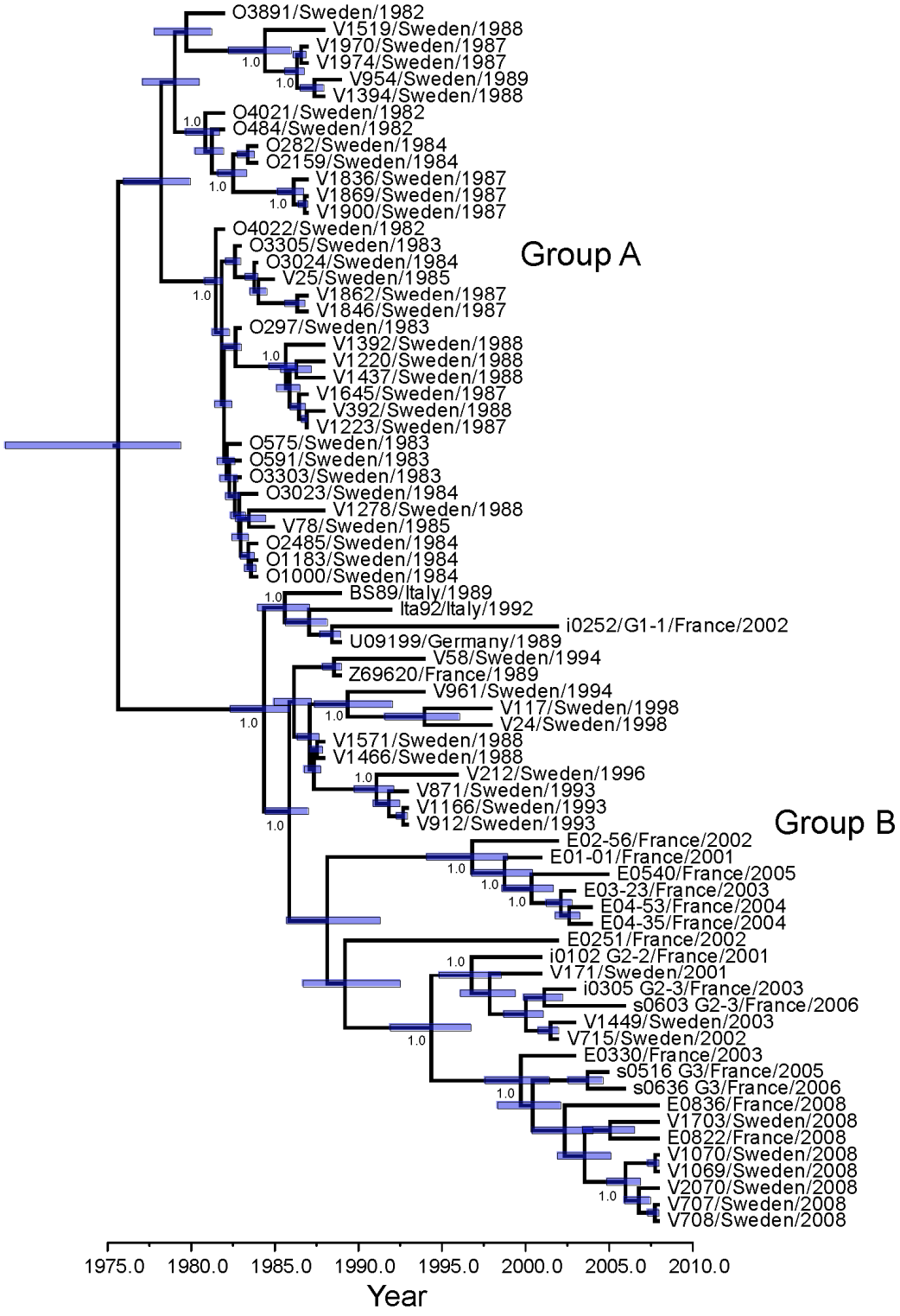

Figure 3.2 MCC tree estimated for the 54 Swedish and assorted European EBHSV samples. The country and year of collection are shown for each sample, while reference sequences retrieved from GenBank have their accession number indicated. Posterior probabilities > 0.9 are shown for selected nodes. Tip times reflect the time (year) of sampling while node bars depict the 95 % HPD values on node times.

The nine EBHSV samples selected for the antigenic study presented five distinct antigenic profiles on the basis of differences in reactivity of four MAbs (O282/Sweden/1984; O1000/Sweden/1984 ≈ V1223/Sweden/1987 ≈ V1519/Sweden/1988 ≈ V24/Sweden/1998 ≈ Ita92/Italy/1992 ≈ Bs91/Italy/1991; V1571/Sweden/1988; V58/Sweden/1994; V708/Sweden/2008 ≈ V2070/Sweden/2008; Table 3.3). The loss of reactivity in MAbs 4E3, 1H1 and 6F9 in some samples from Group B results from epitope modification, probably due to the substitution of one or more amino acids responsible for epitope conformation.

Table 3.3 Antigenic results for the 11 monoclonal antibodies tested. Ita92/Italy/1992 and Bs91/Italy/1991: EBHSV positive controls. Black squares: full reactive; dark grey squares: medium reactive; light grey squares: low reactive; white squares: not reactive. Location of each strain within the major groups of the phylogenetic tree is indicated.

Sample	1C5	4A4	3H6	5F5	2B2	3D6	1F8	1G8	4E3	1H1	6F9	Group
O282/Sweden/1984	■	■	■	■	■	□	■	■	■	▫	▪	A
O1000/Sweden/1984	■	■	■	■	■	■	■	■	■	■	■	A
V1223/Sweden/1987	■	■	■	■	■	■	■	■	■	■	■	A
V1519/Sweden/1988	■	■	■	■	■	■	■	■	■	■	■	A
V1571/Sweden/1988	■	■	■	■	■	▪	■	■	■	■	□	B
V58/Sweden/1994	■	■	■	■	▪	■	▫	■	■	□	□	B
V24/Sweden/1998	■	■	■	■	■	■	▫	■	■	■	▪	B
V708/Sweden/2008	■	■	■	■	■	■	■	▪	□	□	□	B
V2070/Sweden/2008	■	■	■	■	■	■	■	▫	□	□	□	B
Ita92/Italy/1992	■	■	■	■	■	■	■	■	■	■	■	B
Bs91/1991	■	■	■	■	■	■	■	■	■	■	■	-

Strains in Group A (1984–1988) exhibited relatively conserved antigenic profiles, being fully reactive for all MAbs but 3D6 (Table 3.3; Fig. 3.2). In contrast, strains from Group B, collected in a range time of 20 years (1988–2008), showed a progressive variation in antigenic profile. Indeed, the two strains from 2008 presented the most distinct antigenic profile comparing to the original, likely as a consequence of fixation of mutations that caused the loss of reactivity in MAbs 1H1, 6F9 and, finally, 4E3.

Since EBHSV does not grow *in vitro*, the classical approach of producing escape mutants to map the epitopes is not possible. However, comparison of the antigenic profiles and the genetic differences between the strains allowed us to identify the location of some MAbs epitopes. For MAbs 1C5, 4A4, 3H6 and 5F5 no variation in reactivity was observed among strains, such that we could not predict the epitopes recognized. For

MAbs 2B2, 1G8, 4E3 and 6F9, several amino acid substitutions could be responsible for the antigenic variation, but none could be confidently associated with epitope location.

Prediction of epitopes was possible for MAbs 3D6, 1F8 and 1H1 (Fig. 3.3), and further supported by their location in exposed regions of the P2 subdomain of the capsid (Fig. 3.4A). A complete loss of binding of MAb 1H1 is associated with the S383N mutation, as all five 1H1 positive strains have a serine at position 383, whereas 1H1 negative strains have an asparagine. This is likely due to the bulky size of the asparagine extending outward compared to that of serine. In addition, the L378T mutation might interfere with 1H1 reactivity, since strain O282/Sweden/1984 presents a lower reactivity for this MAb. According to this and our capsid structural model, for MAb 1H1 we could demonstrate the associated conformational epitope (Fig. 3.4B). However, this should be treated with caution as the L378T mutation is not associated with a complete loss of reactivity. MAb 1F8 was not fully reactive for strains V58/Sweden/1994 and V24/Sweden/1998. Although no unique substitutions occur in these strains compared to the other strains tested, the mutations at two adjacent positions (T302A and S304G) could independently cause loss of the epitope. With respect to 3D6, the total loss of the epitope in the strain O282/Sweden/1984 might be related with the S408N mutation, since the serine is present only in this strain.

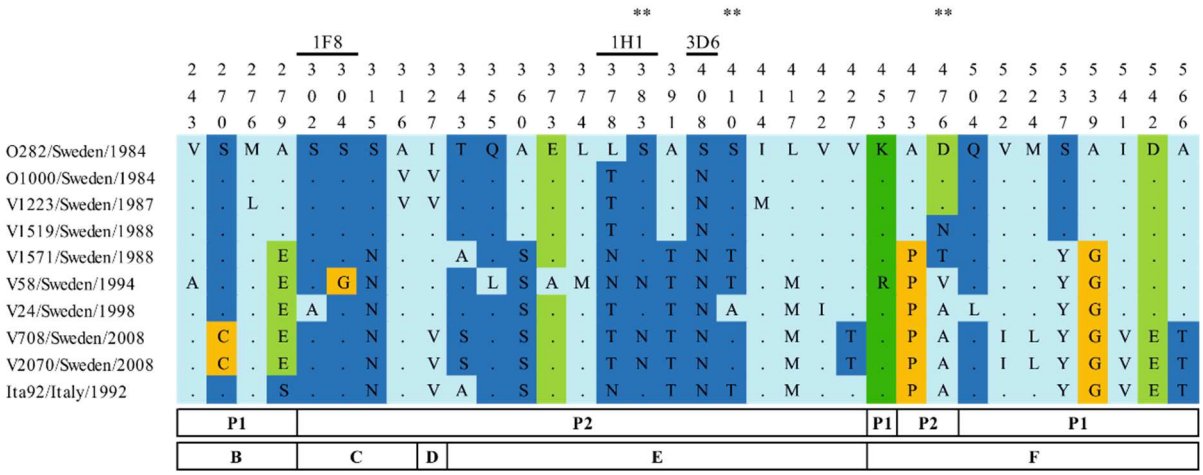

Figure 3.3 Amino acid alignment of the P domain of the nine strains used for the antigenic analyses. Numbers on the top of the figure correspond to the variable amino acid positions found between strains; (.) means identity with the reference sequence. Location of the predicted MAbs epitopes is indicated at the top. Positions under putative positive selection are shown by stars. Amino acids are coloured based on their properties: light blue for hydrophobic amino acids (I, L, A, V, F, Y, M, W); dark blue for amino acids polar uncharged (N, T, Q, S); dark green for amino acids positive charged (R, H, K); light green for amino acids negative charged (D, E) and orange for amino acids with special characteristics (G, P, C). Regions are according to [30] and [31].

Our analysis of site-specific selection pressures acting on VP60 revealed codons 383, 410 and 476 to be under putative positive selection; codons 383 and 410 had a posterior probability > 0.95 for positive selection under the REL and FUBAR models; codon 476 had a posterior probability of 1 for REL, a *p*-value of 0.001 for FEL, and a posterior probability > 0.95 for FUBAR. No episodic diversifying selection was observed on any individual branch. Assuming the organization of *Caliciviridae* genomes proposed by Neill [31], codons 383 and 410 are located in region E of the capsid while codon 476 is located in the region F. For codon 383 two different amino acids – S or N – are observed, but the physiochemical properties are maintained. For codon 410 five different amino acids are found (A, T, S, P and N); interestingly, this codon can be part of a putative N-glycosylation motif. For codon 476 eight different amino acids were found in the sequences studied (A, E, T, S, D, V, G and N); although the physiochemical properties might change with these mutations, the phenotypic effect is uncertain. According to our model of the capsid structure, the positively selected codons are located at exposed regions of the capsid P domain (Fig. 3.4C).

5. Discussion

Despite an increasing interest in calicivirus evolution [32-34], few studies have considered EBHSV even though it is an important agent of disease in Europe. The first recognized and diagnosed EBHSV outbreak occurred in the early 1980s in Sweden [1]. However, there are indications of the presence of the virus within hare populations since at least 1976 [16-18].

Our phylogenetic analysis revealed striking evolutionary patterns in EBHSV. A single viral lineage was apparently dominant at the time of EBHSV emergence (Group A; Fig. 3.2). This lineage spread throughout Sweden, evolving in separated genetic sub-groups that clustered in distinct geographical areas. Group A persisted until 1989 when it was seemingly replaced within a few years by a new lineage (Group B) that emerged in the mid-1980s. While Group A included only Swedish samples from 1982 to 1989, Group B included Swedish samples from the late 1980s and all the Swedish samples from the 1990s and 2000s, along with the other European samples (Italy, France and Germany). Thus, this lineage contains strains detected in several European countries, suggesting that an increased fitness may have enabled it to diffuse over a wider geographic area, at the same time out-competing the pre-existing Group A viruses. This evolutionary pattern resembles that observed for the antigenic variant of RHDV (RHDVa)

in several countries associated with domestic rabbit production, where it replaced earlier RHDV strains [35,36].

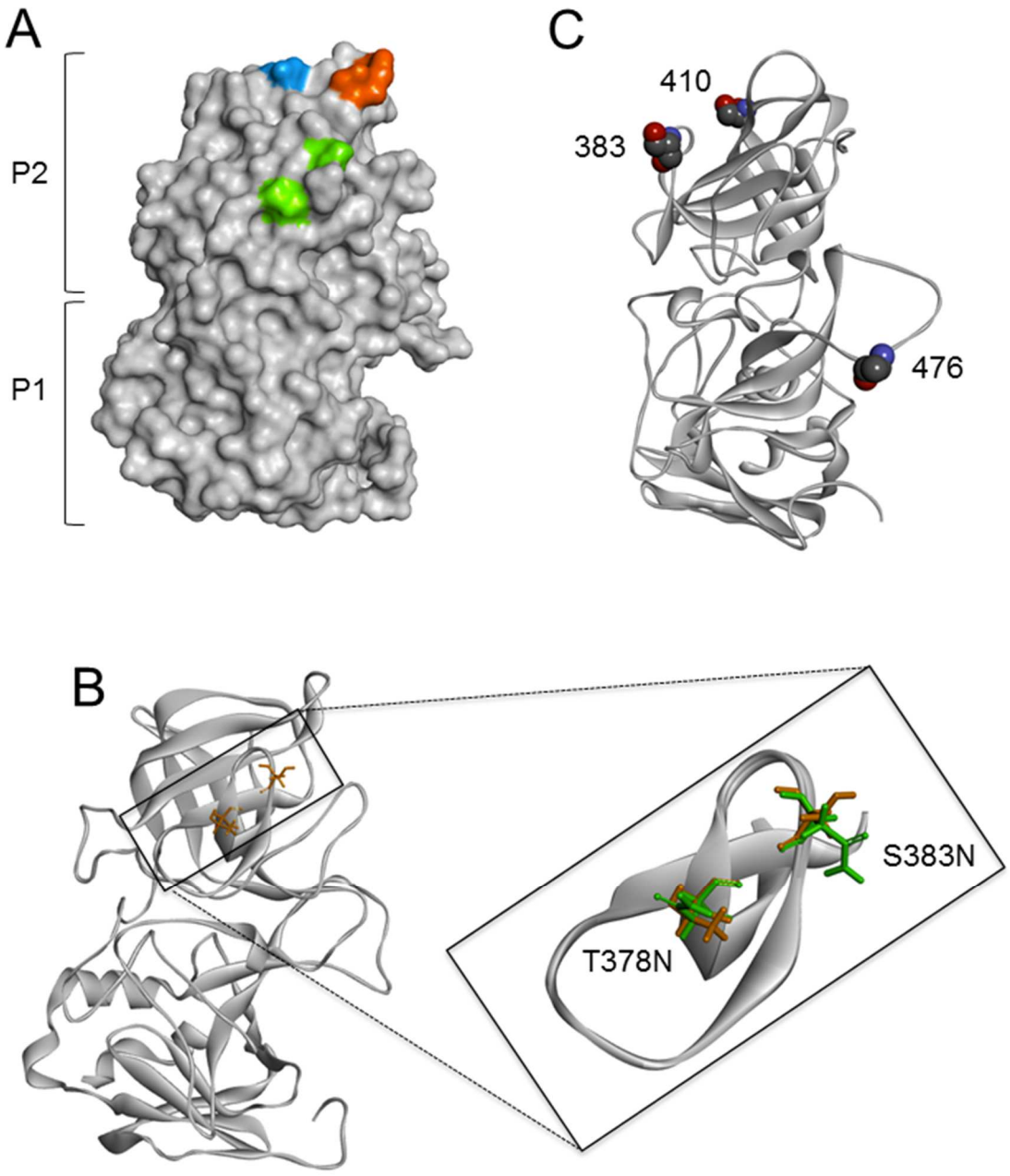

Figure 3.4 Molecular model of the P subdomain of EBHSV capsid protein VP60. (A) Positions of the putative epitopes identified for MAbs 1F8 (red), 1H1 (green) and 3D6 (blue). **(B)** Details of the conformational epitope defined by Mab 1H1 involving positively selected site 383. The two amino acids that modulate MAb binding are highlighted in orange for the recognized O1000/Sweden/1984 strain and in green for the non-recognized V58/Sweden/1994 strain. **(C)** Location on exposed loops of the P domain of putative positively selected sites (codons 383, 410 and 476). The S domain positioned below the P1 subdomain is not shown, since it did not harbor positively selected sites or mutations affecting MAbs binding.

Antigenic variation in EBHSV is observed from as early as 1984, with strains presenting distinct antigenic profiles circulating in the same year. This variation is also observed in 1988, coincident with co-circulation of Groups A and B strains (V1519/Sweden/1988 and V1571/Sweden/1988; Fig. 3.3). In contrast, similar antigenic profiles exist in both groups, but while the phylogenetic analysis is based on the entire capsid sequence, the antigenic analysis only shows differences at specific locations (i.e., within epitopes). The lack of reactivity for the MAbs 4E3, 1H1 and 6F9 that characterizes the antigenic profiles of the most recent Group B strains, makes it possible that the successful persistence of lineage B and the replacement of lineage A is due to a change in antigenicity among these lineages (Fig. 3.3). Indeed, a relationship between antigenic variation and emergence of new outbreaks is well-established for other caliciviruses, such as GII.4 norovirus [37-41].

According to our prediction of the EBHSV secondary structure and considering the similarities with the RHDV atomic model [30], the positively selected codons 383, 410 and 476 are located in exposed loops of P2 and P1 subdomains of VP60, suggesting that, as for RHDV [42], they are associated with host immune pressure. Codon 410 is also located within a putative N-glycosylation motif; a similar observation was made for RHDV and an association with virulence was hypothesized [42]. Interestingly, position 383 appears to dictate reactivity of MAb 1H1, strongly suggesting that it corresponds to a major epitope under immune pressure. Our prediction is supported by a previous study on Italian strains [28]. For MAb 1F8, the amino acids at positions 302 and 304 correspond to the epitope recognized by this MAb. Both T>A and S>G correspond to a loss of the OH group that seems to be associated with the lack of reactivity. Barbieri [28] observed a T>S change at position 302, but the reactivity is not affected since the OH group is maintained. The epitope recognized by MAb 1F8, but also by MAb 3D6, is located in the P2 subdomain, which is the most exposed region of the calicivirus capsid and a highly variable region, being involved in the interaction with host antibodies and histo-blood group antigens [30,43]. It is likely that the changes on the EBHSV capsid influence receptor binding and potential host resistance as described in RHDV [44].

The mean rate of nucleotide substitution for EBHSV estimated in this study, which ranges from 2.6 to 3.6 x 10^{-3} subs/site/year, is relatively high for an RNA virus [45,46], although similar to that recently observed for RHDV in Australia where the virus was introduced as a novel biocontrol agent in 1995 [47]. Under this rate the estimated tMRCA for EBHSV is between 1969 and 1980 (95 % HPD values) which is concordant with its circulation a few years before the original diagnosis in 1980. This time-scale is further supported by (i) the reports of hares that died with lesions consistent with EBHS in

England in 1976, (ii) the identification of EBHSV in samples collected in the 1970s, and (iii) the detection of anti-EBHSV antibodies in sera of hares collected previously [16-18]. Finally, the phylogeographic results reveal a significant clustering by country and Swedish county of origin, such that there are constraints to viral traffic both within Sweden and more broadly across Europe. This sites in contrast to the lack of geographical structure and rapid spatial spread of the related RHDV in some European countries such as Portugal, Spain and France [32,48-50].

In sum, our results show clear replacement of Group A by Group B strains that is likely to be selectively mediated and which may at least in part be associated with antigenic variation. The position of Italian, German and French strains within this Group highlights the successful dispersal and persistence of this lineage throughout Europe. Preliminary data indicate that this lineage is still dominant in Europe and hence merits continued monitoring.

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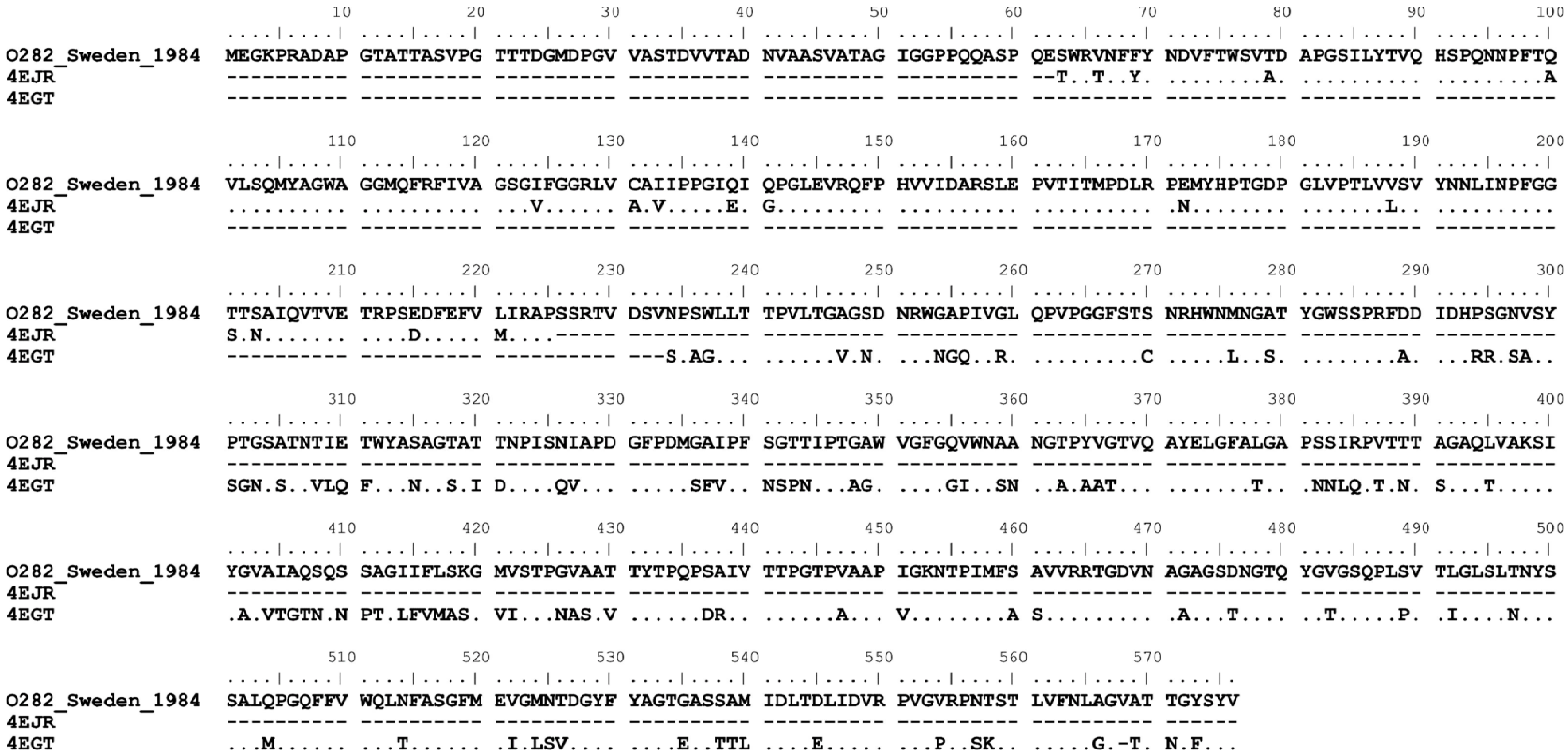
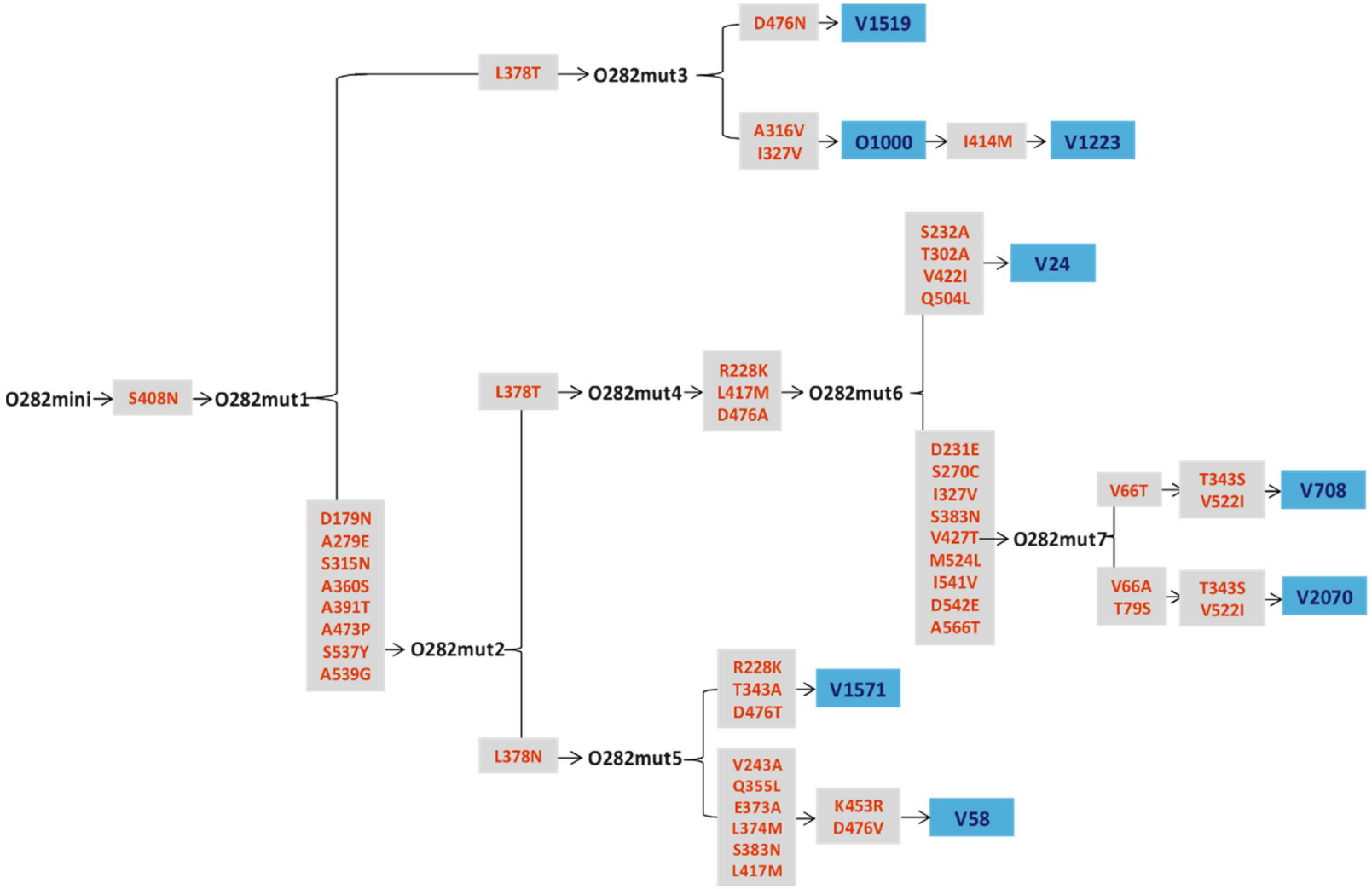
Supplementary Figure 1. Sequence alignment of the domain S and P of the major capsid protein of RHDV (PDB codes: 4EJR and 4EGT, respectively) and EBHSV strain O282/Sweden/1984.



Supplementary Figure 2. Scheme of the mutations from O282/Sweden/1984 to all the other strains used in the model.



A new attachment factor in the *Lagovirus* genus: the European brown hare syndrome virus (EBHSV) uses *N*-acetylglucosamine terminated O-glycans to attach to host cells

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1. Abstract

The European brown hare syndrome virus (EBHSV) is a *Lagovirus* (family *Caliciviridae*) responsible for a hemorrhagic disease affecting hares (*Lepus europaeus* and *L. timidus*). This highly pathogenic virus is similar to rabbit hemorrhagic disease virus (RHDV). The first contact between RHDV and its host, *Oryctolagus cuniculus*, is established via carbohydrates present on the epithelial cells of the upper respiratory and digestive tracts, namely the A, B and H type 2 antigens. The phylogenetic proximity of the two viruses, as well as the similar epidemiological course of the diseases, might foresee the same pathways of virus entry for EBHSV. We tested EBHSV binding to synthetic sugars, and the effect of the removal of key carbohydrates from the tissues on EBHSV attachment. Our results show that EBHSV does not use the same glycans as RHDV, but it seems to prefer GlcNAc-terminated O-glycans to establish the first contact with host cells. These data is central for understanding EBHSV infection cycle.

2. Introduction

European brown hare syndrome virus (EBHSV) is the etiological agent of European brown hare syndrome (EBHS), a fatal disease affecting the European brown hare (*Lepus europaeus*) and the mountain hare (*Lepus timidus*). The disease was first identified in Sweden in 1980 [1] and thereafter in many European countries, becoming endemic in wild hare populations. EBHS causes severe hepatic necrosis and dysfunction in several organs [1], usually leading to death 3-4 days after infection [2]. The mortality rates are highly variable, but can reach up to 100 % in farms, albeit young hares do not

develop the disease [3]. An evolutionary study focused on EBHSV revealed that the early Swedish strains disappeared and that the other European strains are derived from a second group of isolates [4].

EBHSV is a small, non-enveloped, positive-sense, single-stranded RNA virus, with ~ 7.4 kb in length. Viral particles were first identified in the liver of dead hares in 1989 [5]. These particles were recognized as closely related to those of rabbit hemorrhagic disease virus (RHDV) [5], a virus that also emerged in the 1980s [6] and that is responsible for the decline of European rabbit (*Oryctolagus cuniculus*) populations. These two viruses share extensive similarities [7-9], although with a genetic distance of ~ 30 % [10], and were assigned to the genus *Lagovirus*, family *Caliciviridae* [11].

The hosts of these two viruses are both members of the order Lagomorpha. In Europe, three lagomorph genera are found, with *Sylvilagus* as a non-native species, since it was introduced in several occasions in the 1960s and 1970s [12]. The genera *Oryctolagus* and *Lepus* separated ~ 12 million years ago [13] and are represented in Europe by seven species: *O. cuniculus*, *L. europaeus*, *L. timidus*, *L. capensis* (only in Sardinia), *L. corsicanus*, *L. granatensis* and *L. castroviejoi* [14]. Although *L. granatensis* has a contact zone with *L. europaeus* (EBHSV susceptible), no cases of EBHSV had been reported in this species. Strikingly, a spillover infection of RHDV was described in *L. granatensis* [15].

Virus attachment to host cell receptors is a key factor to successfully infect the host and initiate the virus life cycle. This acquires special importance on virus evolution since changes in receptor binding motifs impose constraints on viruses, which ultimately can lead to the emergence of new strains with different receptor requirements. RHDV attaches to histo-blood group antigens (HBGAs) of the upper respiratory and digestive tracts to start infection [16] and its ability to bind to the A, B and H type 2 epitopes is dependent on the virus' genogroup [17]. For example, while the Iberian G1 has affinity for A, B and H, the French G2 does not show affinity for the A antigen, but only for the B and H antigens [17].

More recently, a dominant-negative mechanism was shown for the α1,2fucosyltransferase Sec1 over Fut1, a key enzyme for the synthesis of RHDV ligands in the host. The study also showed an extensive gene conversion involving the genes of this family for the leporids analyzed [18]. This study represented a significant step forward in understanding the RHDV infectious process and the rabbit resistance mechanisms. On the contrary, little is known about how EBHSV initiates this process. Given the similarities between RHDV and EBHSV regarding morphology, genome

organization and antigenicity, we investigated the attachment requirements of EBHSV to the host and the possible role of HBGAs in this process. We further investigated if any dissimilarity between EBHSV binding to *L. europaeus* and *L. granatensis* could explain the absence of EBHSV infection in the latter.

3. Methods

Ethic statement

Tissue samples were collected from two hares shot by hunters from Idanha, Portugal (*L. granatensis*), one hare reared in captivity and humanely euthanized (*L. granatensis*) and four *L. europaeus* from Spain also reared in captivity. Samples of trachea and duodenum from two additional *L. europaeus* hunted in western France were used for immunohistochemistry. Rabbit duodenums were collected from freshly hunted wild rabbits from the Pyrénnées Orientales, France. These samples had previously been phenotyped for HBGAs [17].

An anti-EBHSV polyclonal antibody was generated by serial inoculation of two rats with EBHSV virus-like particles (VLPs) produced from the sequence of a French strain, kindly provided by Ghislaine Le Gall-Reculé (ANSES, Ploufragan, France). Experimentation was performed at the animal experimentation core facility, University of Nantes. Recognition of target VLPs using the generated antibody was confirmed by ELISA. Use of rats was approved by the national ethic review board from the French Ministry of 'Enseignement Supérieur et de la Recherche' (project license number 01322.01). The animal care and use protocol adhered to the European Directive number 2010/063 and to the national French regulation (Décret nº 2013-118 du 1er février 2013 relatif à la protection des animaux utilisés à des fins scientifiques).

Preparation of samples and VLPs

Samples of hare duodenum and trachea were collected and immediately fixed in 10 % buffered formalin and later paraffin-embedded and sectioned. Hare duodenum scrapings were prepared from 1 cm of duodenum for three *L. granatensis* and four *L. europaeus* and ABH phenotyped by ELISA as described in [17].

VLPs of an EBHSV strain were generated using a previously described method [19]. Briefly, recombinant baculoviruses were generated containing the VP60 protein, then VLPs were produced by infection of Sf9 insect cells. VLPs were released from the

infected Sf9 cells by three rounds of freeze-thawing, and then clarified by removal of cellular debris (6,000 x g for 30 minutes) and baculovirus (14,000 x g for 30 minutes). VLPs were partially purified through a 30 % (w/v) sucrose cushion in TNC buffer (50 mM Tris HCl pH 7.4, 150 mM NaCl, 10 mM $CaCl_2$) containing the protease inhibitor leupeptin for 150,000 x g for two hours. The pelleted VLP was resuspended in TNC buffer and further purified by isopynic centrifugation in caesium chloride (150,000 x g for 18 hours). The resultant VLP bands were collected by puncture and the solution containing VLPs was dialyzed against PBS prior to quantification in nanodrop and kept at -20 ºC.

EBHSV binding assays

EBHSV binding to tissues was assessed using ELISA and immunohistochemistry (IHC). For ELISA, Nunc Maxisorp plates were coated with hare scrapings diluted 10^{-5} and 10^{-7} in 100 mM sodium carbonate buffer, pH 9.5, and incubated at 4 ºC overnight. All the following incubations were at 37 ºC in humid atmosphere; samples and antibodies were diluted in sterile PBS; plates were washed three times with 0.05 % Tween 20 in PBS after each incubation. Plates were blocked with 5 % non-fat dry milk diluted in sterile PBS for one hour. The wells were then incubated with EBHSV VLP at 12 µg/mL for two hours, followed by a one-hour incubation with the rat anti-EBHSV serum diluted 1/500. One last incubation with an anti-rat IgG HRP-conjugated 1/1000 for one hour was performed. Finally, TMB was used as substrate and the reaction was stopped after 8 min with 1 M phosphoric acid. Optical density (O.D.) values were read at 450 nm.

For IHC, hare tissue sections were deparaffinized through LMR-SOL baths and rehydrated with ethanol. Endogenous peroxidase activity and non-specific binding were blocked with room temperature baths of 20 min with 0.3 % (v/v) hydrogen peroxide in PBS and of 30 min with 3 % (w/v) bovine serum albumin (BSA) in PBS, respectively. A 10 % (w/v) liver homogenate prepared from a hare that tested positive for EBHSV and diluted 1/5 was incubated with the tissue sections at 4 ºC overnight. Samples and antibodies were diluted in 1 % (w/v) BSA in PBS; after each incubation, slides were washed 3 x 5 min in PBS. A 1/100 dilution of the EBHSV-specific monoclonal antibody (MAb) 5F5 (kindly provided by Lorenzo Capucci) was then incubated with the tissue sections for two hours at 37 ºC. MAb binding was followed by an incubation with biotinylated anti-mouse antibody diluted 1/1000, one hour at 37 ºC. Slides were then incubated with HRP-conjugated avidin D, one hour at room temperature, and finally substrate (AEC kit) was added to the slides. After 25 min, slides were washed as described above and Mayer's hematoxylin was used for contrast staining.

Test of EBHSV binding to histo-blood group antigens

A panel of PAA-conjugated and BSA-conjugated synthetic sugars was used to screen EBHSV binding to HBGAs, according to RHDV binding patterns [16,17]. Sugars were coated at 1 µg/well and incubated overnight at 4 ºC. All subsequent steps were performed as described for EBHSV binding to tissue scrapings, except for the VLP binding step for PAA-conjugated sugars with the incubation steps being performed at 4 ºC.

Enzyme treatments on tissues and EBHSV binding

Rabbit duodenum samples were coated in Nunc Maxisorp plates at 1/100 dilution at 4 ºC overnight. Prior to the blocking step, plates were incubated two hours at 37 ºC with the following enzymes: α-*N*-acetylgalactosaminidase (New England Biolabs), 50 U/well; α-galactosidase from green coffee beans (Sigma), 0.2 U/well; 1,2-α-L-fucosidase from *Bifidobacterium bifidum*, 0.5 µg/well. After incubation with each enzyme, ELISA steps were performed as described above for binding to tissue scrapings. The same protocol was applied for PNGase F (New England Biolabs), but duodenum tissue was from a hare and used at 1/400 dilution; incubation with the enzyme lasted six hours.

For α2-3,6,8 Neuraminidase (New England Biolabs) and *O*-sialoglycoprotein endopeptidase (OSGE; Cedarlane) a slightly modified protocol was adopted. Hare duodenum samples were incubated overnight at 37 ºC with each enzyme and then coated in Nunc Maxisorp plates at 4 ºC overnight at a final dilution of 1/400. After blocking the plate, steps were carried out as described above. A treatment with sodium metaperiodate was also performed, using the same protocol as for A, B and H enzymes. Sodium metaperiodate was added to the wells in concentrations ranging from 1 mM to 100 mM, incubated for one hour at 37 ºC and then, prior to blocking, glycine 1 % in PBS was added to the wells for 30 min.

Printed glycan array (PGA)

Printed glycan array slide fabrication and high-throughput profiling was performed as previously described [20]. Briefly, 360 monomeric glycans as ω-aminopropyl glycosides of 95-98 % purity (Lectinity) were diluted in 300 mM phosphate buffer pH 8.5, containing 0.005 % Tween 20 and printed by robotic pin deposition on *N*-

hydroxysuccinimide activated glass slides (Nexterion Slide H, Schott, Jena, Germany). Glycans were printed at a 50 µM concentration in six replicates. Free *N*-hydroxysuccinimide activated groups were blocked with 50 mM ethanolamine in 50 mM borate buffer at a final pH of 9.2. Slides were then rinsed with deionized water, dried and stored at room temperature in a desiccator. VLPs diluted in PBS containing 0.1 % (v/v) Tween 20 and 3 % (w/v) BSA were incubated on the array slides and gently rocked in a humidified incubator at 37 ºC for two hours. Unbound VLPs were washed with a series of 0.1 % and 0.001 % Tween 20 in PBS. Anti-EBHSV diluted 1/1000 in PBS containing 0.1 % (v/v) Tween 20 and 3 % (w/v) BSA was added and slides were incubated at room temperature in a humidified chamber for 45 minutes and then washed. Bound antibodies were visualized by incubating slides with Cy5-labeled anti-rabbit IgG 1/1000 in PBS/0.1 % Tween 20 at room temperature for 30 minutes. Following further washings, fluorescence signals corresponding to glycan-bound antibodies were measured using an Agilent scanner G2565CA and analyzed using ImaGene analysis software version 7.5 (BioDiscovery, El Segundo, USA). Signals were measured as total signal intensity (medTSI) per glycan and were expressed as median across six intra-array replicates.

4. Results and Discussion

The first step for a successful viral infection is the attachment of the virus to host cell surface receptors. This virus-host interaction is of extreme importance, dictating tissue tropism and pathogenicity, and might be mere electrostatic and non-specific, albeit allowing the virus the establishment of a contact point to further attract specific receptors responsible for cell entry [21]. Several studies on rabbit hemorrhagic disease virus (RHDV) entry disclosed that histo-blood group antigens (HBGAs) A, B and H type 2 are involved in virus attachment to epithelial cells of the upper respiratory and digestive tracts [16,17]. Despite the close relationship between RHDV and European brown hare syndrome virus (EBHSV), the means used by the latter to attach to host cells are unknown.

Our first approach to understand EBHSV attachment was to determine the binding pattern on tissues of the upper respiratory and digestive tracts. We observed that EBHSV binds to the epithelial layer of trachea and to the surface layer of the duodenal mucosa (Lieberkühn crypts) (Fig. 3.5), as has been shown for RHDV [16]. No differences in the binding pattern were observed between the two hare species used in this study, *L. europaeus* and *L. granatensis*, despite their apparent different susceptibility to EBHSV.

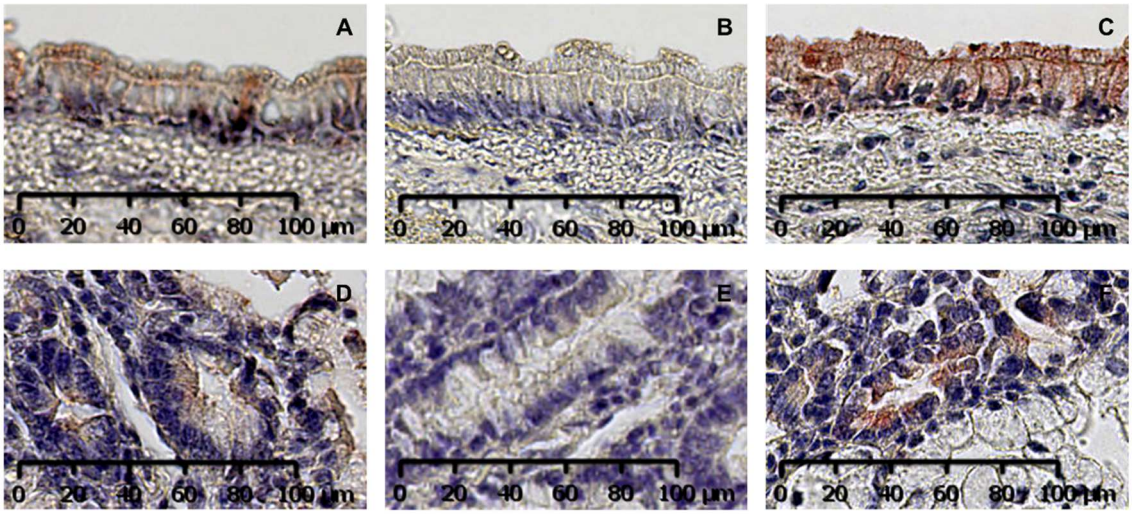

Figure 3.5 EBHSV binding to hare trachea and duodenum. The virus binds to the epithelial layer of trachea **(A)** and to the Lieberkühn crypts **(D)** in the duodenum, as the other member of the genus, RHDV. **(B)** and **(E)** are the negative controls (same treatment as the other slides, but omitting the incubation with the virus) for trachea and duodenum, respectively. **(C)** and **(F)** represent RHDV binding to rabbit trachea and duodenum, respectively, and are shown for comparison. Scale bar is indicated for each case.

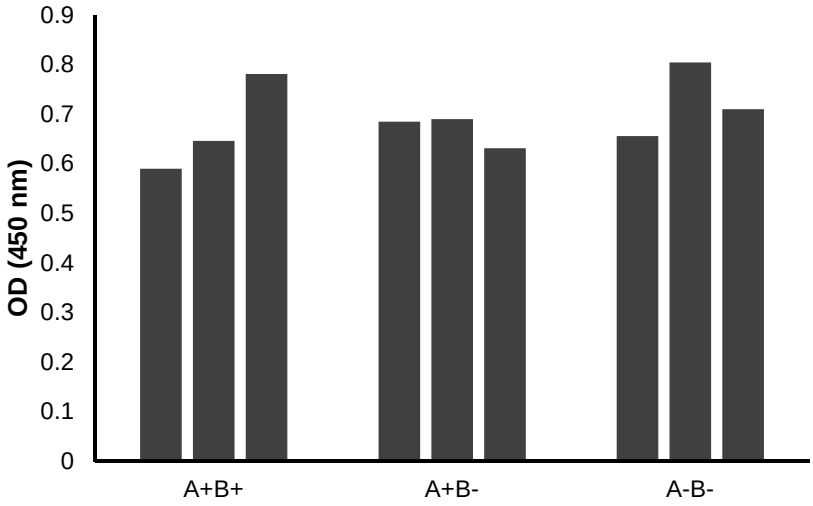

Figure 3.6 EBHSV binding to different ABH phenotypes. Bars represent values obtained for binding to tissue extracts from three individual rabbits for each ABH phenotype (A^+B^+, A^+B^- and A^-B^-). OD: optical density.

As all phenotyped hares revealed to be negative for the presence of the B antigen (data not shown), ABH phenotype-dependent binding of EBHSV was tested on rabbit tissues. For this, EBHSV binding to rabbits with different phenotypes (A^+B^+, A^+B^- and A^-B^-) was performed by ELISA. No significant preference for any phenotype or trend was observed (Fig. 3.6). When tissues were treated with specific enzymes that remove the

α-linked *N*-acetylgalactosamine and galactose (for A and B antigens, respectively) and the α1,2-linked fucose residues (for H antigen), no significant decrease in binding was observed for EBHSV (Fig. 3.7), reinforcing the absence for a preference of a specific ABH phenotype/antigen.

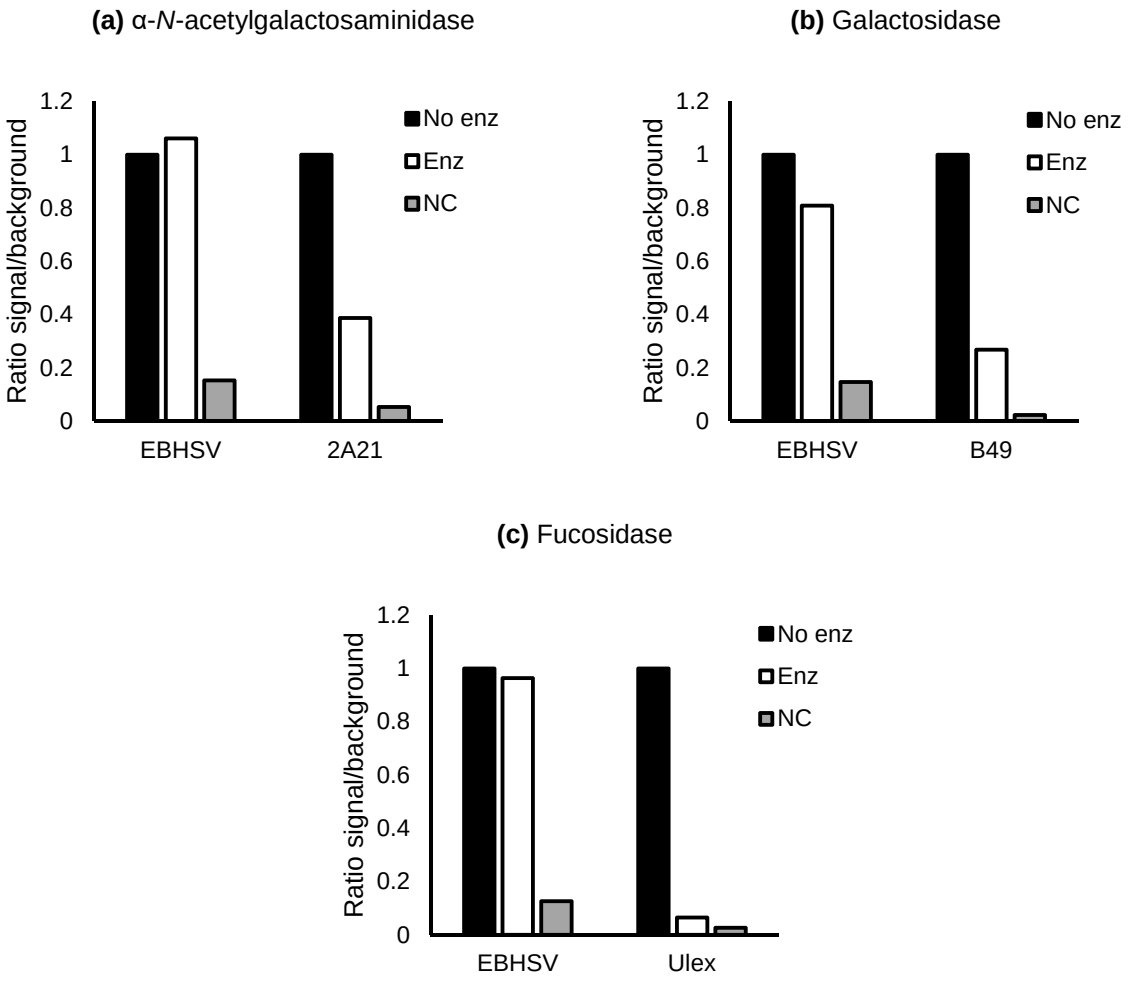

Figure 3.7 EBHSV binding to tissues following enzyme treatments. (a) α-*N*-acetylgalactosaminidase treatment, specific for A antigen; **(b)** galactosidase treatment, specific for B antigen; **(c)** fucosidase treatment, specific for H antigen. For each enzyme, a control of the enzyme activity was used: the anti-A antibody for α-*N*-acetylgalactosaminidase; the anti-B antibody B49 for galactosidase and the anti-H Ulex for fucosidase. Negative controls (NC) were performed by incubating the wells as the treatments but without the VLP and positive controls (No enz) were performed by incubating the wells as the treatments but without the enzyme; Enz: OD results for treated wells.

A range of synthetic sugars was then selected for testing the EBHSV binding, according to previous data on RHDV [16,17]. Strikingly, EBHSV did not bind to any of the tested sugars (data not shown). Thus, as no significant decrease in tissue binding after enzyme treatments and no binding to synthetic sugars that are used by RHDV to

attach epithelial cells were observed for EBHSV, we hypothesized that EBHSV uses alternative attachment factors, rather than using HBGAs. This led us to perform the glycan arrays experiments that allow to assay a large number of glycan structures. The glycan arrays revealed a significant binding signal to terminal *N*-acetylglucosamine residues, and to heparin (Table 3.4). Consistent with the above described results, no significant binding was observed on glycans of the fucosylated histo-blood group type that constitute ligands for RHDV. Likewise, no binding was recorded to glycans containing sialic acid residues that constitute ligands for many viruses. Therefore, EBHSV seems to use alternative glycans as attachment factors to start the infectious process.

Table 3.4 Glycan array results. Only glycans with a ratio signal/background higher than 10 are shown.

Common (short) name	Structure	Signal (S)	Background (B)	Ratio (S/B)
Chitohexaose (Ch6)	(GlcNAcb1-4)6-sp4	16673	722	23.1
Chitopentaose (Ch5)	(GlcNAcb1-4)5b-sp4	18697	847	22.1
Heparin	Heparin (NH4+ salt)	47739	3170	15.1
Chitobiose-Asn (Ch2-Asn)	GlcNAcb1-4GlcNAcb-Asn	17440	1553	11.2

Glycosaminoglycans (GAGs) usually exist as O-linked chains of proteins. Heparin is structurally similar to another GAG, heparan sulfate. Virtually, all mammalian cells express heparan sulfate, a complex polysaccharide composed by repeating disaccharide units of glucoronic acid or iduronic acid linked to glucosamine, that can display a remarkable range of different sequence motifs and high structural diversity [22]. Interactions of heparan sulfate with proteins are established via hydrophobic interactions and hydrogen bonding [23-25], but mainly through electrostatic interactions of its negatively charged sulfate with basic amino acids [25,26].

Heparin-binding domains have been shown to contain consensus sequence motifs, such as XBBXB, XBBBXXBX or XBBXBX, where B is a basic amino acid and X is a neutral or hydrophobic amino acid. We analyzed the publicly available sequences of EBHSV capsid protein and although this protein does not possess any of these motifs, it has a ^{464}RR^{465} motif that is exposed according to the structural model proposed for EBHSV [4]. Nevertheless, the binding pattern of EBHSV on tissue sections does not match with the distribution of heparan sulfate (Fig. 3.5). In addition, assay of the glycan specificity of VLPs from several other related viruses, including RHDV, showed binding to heparin, alongside the well described binding to fucosylated histo-blood group antigen

motifs (data not shown). The involvement of heparan sulfate in binding and infection by these viruses therefore remains to be assessed.

To further delineate the nature of the glycans recognized by EBHSV, enzyme treatments were performed. When removing N-glycans or sialic acids, through treatments with PNGase F or Neuraminidase, respectively, EBHSV binding to tissues is not affected (Fig. 3.8a,b). The same is observed when incubating the tissues with increasing amounts of sodium metaperiodate, which degrades carbohydrate structures by cleaving C-C bonds (Fig. 3.8d). However, when the tissue was treated with the *O*-sialoglycoprotein endopeptidase (OSGE) enzyme, EBHSV binding was significantly reduced (Fig. 3.8c). This enzyme removes proteins that have O-linked sugars on their surface, indicating that the motifs recognized by EBHSV are O-glycans carried by mucin-type proteins. Interestingly, a structural analysis of mucin-type O-glycans from the duodenum of rabbits revealed the presence in limited amounts of GlcNAc-terminated motifs [17]. A similar analysis has not been performed in hares, but suggests the presence of potential EBHSV ligands on O-glycans from the small intestine of a lagomorph species. EBHSV and RHDV, the two viruses of the family *Lagovirus*, are very similar in terms of morphology, antigenicity, genome organization and disease progress [7,9,27,28] despite their ~ 30 % genetic distance [10]. Therefore, the reason why EBHSV uses different sugars to attach host cells remains unclear.

The majority of caliciviruses uses carbohydrate ligands to attach host cells, but the type of carbohydrate varies across genera. For example, several human norovirus strains, mainly those derived from GII strains, were also reported to bind to heparan sulfate [29]. Heparan sulfate is a primary or co-receptor for viruses from other families, for example, adeno-associated virus, family *Parvoviridae* [30], human immunodeficiency virus, family *Retroviridae* [31], herpes simplex virus, family *Herpetoviridae* [32], and filoviruses [33]. However, it is unknown if heparan sulfate, and glycosaminoglycans in general, play a role in caliciviruses attachment and entry. Caliciviruses such as noroviruses can also use HBGAs to attach to host cells [34], and this has clearly been associated with infection [35]. Likewise, RHDV binds to fucosylated glycans and this too has been associated with infection [16-18]. However, some murine norovirus strains appear to require binding to sialic acid containing N- and O-glycans whilst others bind to N-glycans in a neuraminidase insensitive manner [36].

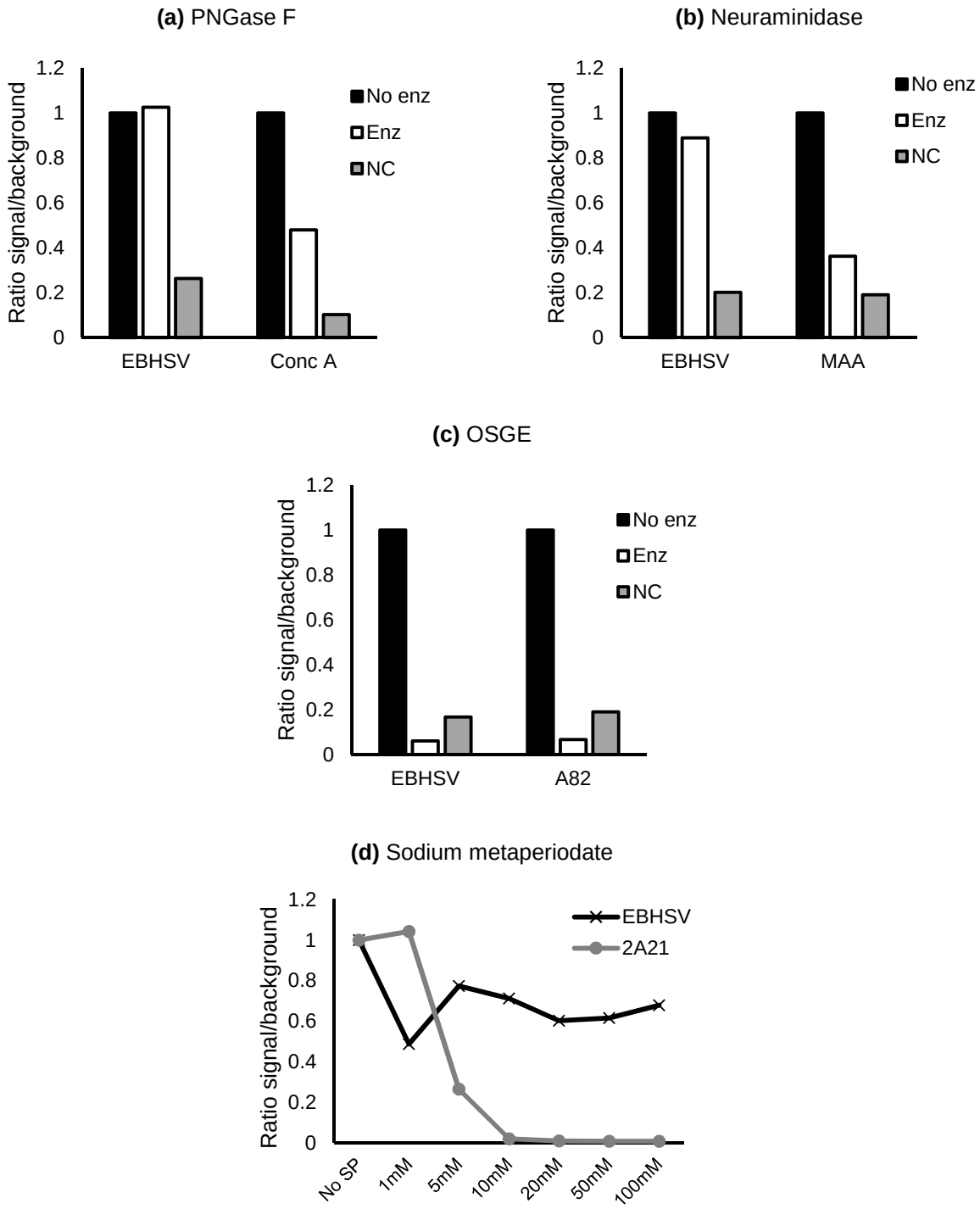

Figure 3.8 Enzyme treatments on tissues followed by EBHSV binding. Lectins *Concanavalin A* (Conc A) and *Maackia Amurensis* Lectin II (MAA) were used to evaluate PNGase F **(a)** and Neuraminidase activity **(b)**, respectively. Antibody A82 was used with the same purpose for OSGE enzyme **(c)**. Antibody 2A21 recognizes A antigen and was used as control for the sodium metaperiodate activity **(d)**. Controls were included as described in Fig. 3.6; Enz: OD values for treated wells.

A common theme is that all caliciviruses tested so far bind to some type of glycans, but that distinct types may be recognized by different viruses. These differences in the type of glycans used for host attachment might also correlate with the different

hemagglutination properties of EBHSV and RHDV. Indeed, for EBHSV the hemagglutination test requires some modifications when comparing to the protocol used for RHDV, such as mixing the samples with chloroform, carrying the test at 4 ºC and at pH 6.5 [7]. As GAGs and terminal GlcNAcs are not present in red blood cells, EBHSV is not able to agglutinate them, at least at the same extent as RHDV.

Our study identified a GAG and GlcNAc-terminated O-glycans as potential new attachment factors in the *Lagovirus* genus and improved our knowledge on how the virus enters into host cells and other factors related to virus-host interactions. This data will additionally provide information on the evolution of EBHSV.

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Chapter 4

The impact of host shifts in *Lagovirus* evolution

Paper VIII. Lopes AM, Marques S, Silva E, Magalhaes MJ, Pinheiro A, Alves P, Le Pendu J, Esteves PJ, Thompson G, Abrantes J: **Detection of RHDV strains in the Iberian hare (*Lepus granatensis*): earliest evidence of rabbit lagovirus cross-species infection**. *Veterinary Research* 2014, **45**:94.

Paper IX. Lopes AM, Lora M, Le Moullac-Vaidye B, Galanina OE, Breiman A, Strive T, Marchandeau S, Le Gall-Reculé G, Bovin NV, Ruvoën-Clouet N, Esteves PJ, Abrantes J, Le Pendu J: **Leporids tissue binding and glycan specificities of the new RHDV variant and non-pathogenic related viruses**. In preparation.

Detection of RHDV strains in the Iberian hare (*Lepus granatensis*): earliest evidence of rabbit lagovirus cross-species infection

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1. Abstract

Rabbit hemorrhagic disease virus (RHDV) is a highly lethal *Lagovirus*, family *Caliciviridae*, that threatens European rabbits (*Oryctolagus cuniculus*). Although a related virus severely affects hares, cross-species infection was only recently described for new variant RHDV in Cape hares (*Lepus capensis mediterraneus*). We sequenced two strains from dead Iberian hares (*Lepus granatensis*) collected in the 1990s in Portugal. Clinical signs were compatible with a *Lagovirus* infection. Phylogenetic analysis of the complete capsid gene positioned them in the RHDV genogroup that circulated on the Iberian Peninsula at that time. This is the earliest evidence of RHDV affecting a species other than European rabbits.

2. Introduction, methods and results

Wild and domestic European rabbits (*Oryctolagus cuniculus*) are severely affected by rabbit hemorrhagic disease (RHD), an acute hepatitis that causes death within 48–72 h after infection [reviewed in 1]. RHD was first reported in 1984 in China and two years later was described in Italy, spreading to several European countries afterwards [reviewed in 1]. On the Iberian Peninsula (IP), the first reports of RHD date from 1988 [reviewed in 1]. The etiological agent of the disease, rabbit hemorrhagic disease virus (RHDV), belongs to the *Caliciviridae* family, genus *Lagovirus*. The other member of this genus, European brown hare syndrome virus (EBHSV), causes the European brown hare syndrome (EBHS) in European and mountain hares (*Lepus europaeus* and *L.*

timidus, respectively) [2]. On the IP, EBHSV was first reported in *L. europaeus* in 1995 (Gortázar C, personal communication). RHDV and EBHSV are non-enveloped, positive-sense, single-stranded RNA viruses, with a similar genomic organization and ~ 70 % of nucleotide identity [3]. RHD and EBHS share clinical signs and pathological alterations, including congestion and necrosis of the liver and massive disseminated intravascular coagulation [reviewed in 1].

Two subspecies of *O. cuniculus* are found on the IP, *O. cuniculus cuniculus* in the northeast and *O. cuniculus algirus* in the southwest (Figure 4.1) [4]. Additionally, three hare species inhabit this region: *L. granatensis*, distributed along Portugal, mainland Spain and Majorca; *L. castroviejoi*, restricted to Cantabria; and *L. europaeus*, confined to the Pyrenees (Figure 4.1) [4]. Although EBHS was reported in Spain, there are no reports of the disease in *L. granatensis* and *L. castroviejoi*, both endemic to the IP.

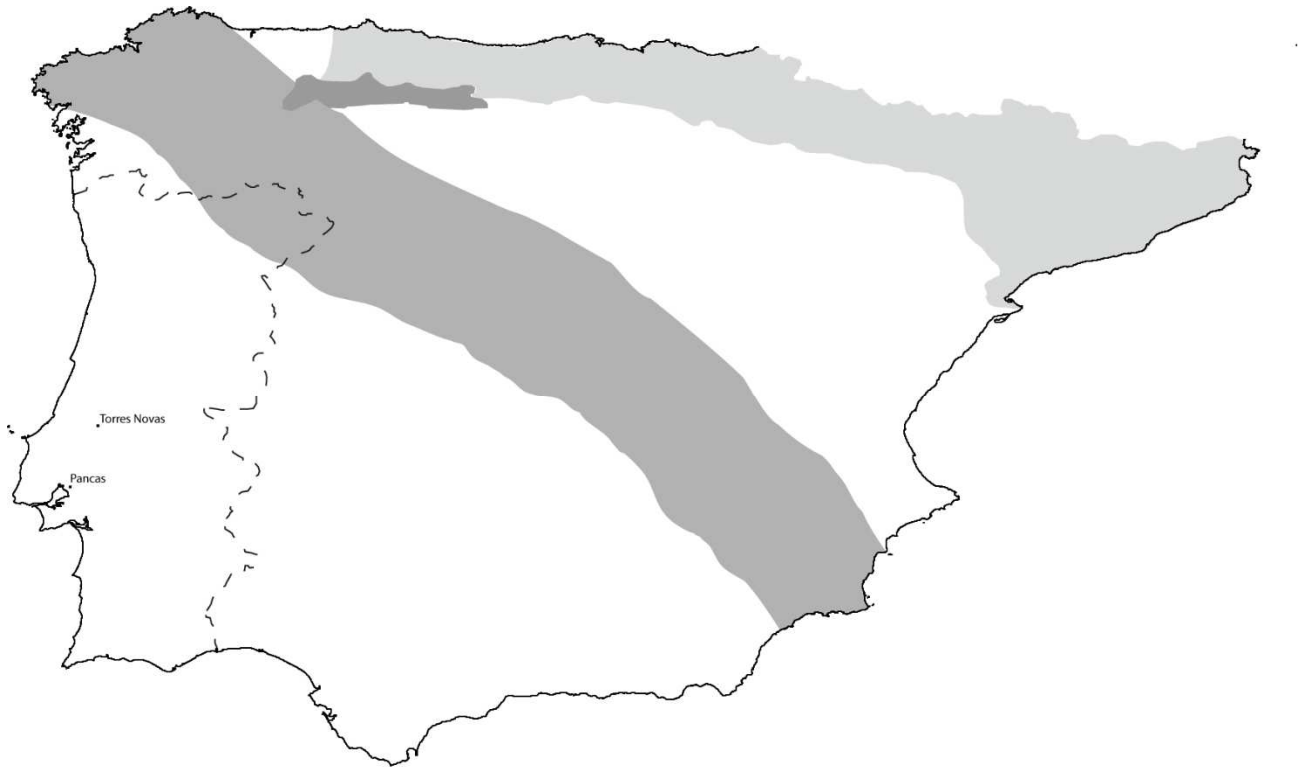

Figure 4.1 Map with the distribution of leporid species on the Iberian Peninsula. Light grey represents the distribution of *Lepus europaeus*. Dark grey corresponds to the distribution of *L. castroviejoi*. White represents the distribution of *L. granatensis*. The European rabbit (*Oryctolagus cuniculus*) is present all over the Iberian Peninsula; the contact zone between *O. c. cuniculus* and *O. c. algirus* (restricted to the southwest region) is in medium grey. The localities where P95 (Torres Novas) and P151 (Pancas) were collected are indicated.

Despite their similarities, RHDV and EBHSV are restricted to their natural hosts, as has also been proven experimentally [5]; however, cross infection was recently described for a new RHDV variant (tentatively named RHDV2 or RHDVb) in *Lepus capensis mediterraneus* in Italy [6]. RHDV2 was originally described in France in 2010 [7], and is antigenically and genetically different from the classical RHDV strains [7,8]. In this study we report the first and earliest evidence of cross-species infection of classical RHDV in the Iberian hare, *L. granatensis*.

Liver samples of two hares found dead in the field were collected in Torres Novas and Pancas, south of Portugal, in 1996 and 1998, respectively (P95 and P151; Figure 4.1). At necropsy, lesions compatible with an infection by a *Lagovirus* were observed. P95 showed congestion of the liver and lungs as well as non-coagulated blood in the chest cavity, trachea and lungs; P151 presented congestion of the kidneys and both the chest and abdominal cavity were filled with non-coagulated blood and presented dark red coloration. In both animals, blood vessels were dilated and filled with non-coagulated blood. Additionally, the livers of five dead rabbits collected in Pancas in November and December-1997 were included in this study (P129, P141-P144). These individuals also exhibited lesions compatible with a *Lagovirus* infection at necropsy: dark red coloration and congestion of the kidneys and spleen; heart in diastole and atria filled with blood; non-coagulated blood in the trachea, chest and abdominal cavity (P143 presented normal abdominal cavity). With the exception of P129, dilated blood vessels filled with non-coagulated blood were observed, as well as a hypertrophic, congested liver. P142-P144 additionally presented congested lungs, filled with non-coagulated blood and P129, P141 and P142 presented nasal hemorrhages.

To avoid any risk of contamination, RNA extraction, cDNA synthesis and PCR amplification of the two hare samples were performed independently from the other samples. Total RNA was extracted with the RNeasy Mini Kit (Qiagen, Hilden, Germany) and reverse transcription was performed using oligo(dT) as primers and Super-Script™ III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). Protocols were performed according to the manufacturer's instructions. The samples were screened for the presence of RHDV and EBHSV by PCR with the primers U38 and EBHS9 as described elsewhere [9] as these primers amplify both RHDV and EBHSV. Sequencing of the fragments revealed that samples were positive for RHDV and negative for EBHSV. Additionally, and to ensure that only RHDV was present, RHDV and EBHSV-specific PCR were also performed (conditions available upon request). Amplification was successful for RHDV, but not for EBHSV. The gene encoding the capsid protein VP60 of RHDV (1740 base pairs) was fully amplified by PCR using 1 µL of the cDNA reaction,

2 pmol of each oligonucleotide, 5 µL of Phusion Flash High-Fidelity PCR Master Mix (Thermo Scientific, Waltham, Massachusetts, USA) and water to a final volume of 10 µL, using several pairs of primers (PCR cycle conditions are available upon request). After purification, PCR products were sequenced on an automatic sequencer ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems, Foster City, CA, USA) with the amplification primers. Sequences were submitted to GenBank under the accession numbers KJ943791 and KJ943792 for the sequences from hares and KJ943793-KJ943797 for the sequences from rabbits. A Blast analysis of the sequences revealed 98 % identity of P95 with strain AST89 (genogroup 1; accession number Z49271) and 97 % identity of P151 with strain 00–08 (Iberian group 3 of genogroup 1 [10]; accession number AJ319594).

The capsid sequences were aligned with publicly available sequences of *Lagovirus* (for simplicity, only part of the available sequences were used), namely RHDV genogroups 1–5 (G1-G5) [11], the antigenic variant RHDVa (G6), RHDV2, the weakly pathogenic MRCV, the non-pathogenic strain RCV-A1, and EBHSV (accession numbers of the sequences used are indicated in Figure 4.2). Furthermore, the alignment was screened for recombination as described elsewhere [12]. The phylogenetic relationships between the samples were estimated in MEGA6 [13], by using a Maximum Likelihood (ML) approach. The support of the nodes was assessed with a bootstrap resampling analysis (1000 replicates). The best-fit nucleotide substitution model defined by the same software (GTR + I + G) was used in the analysis.

The inferred ML relationships were in agreement with those published, with EBHSV, RHDV2 and RHDVa forming well supported groups (bootstrap values of 1; Figure 4.2). Genogroups 1–5 cluster together, with G1, G2 and G3-G5 also forming well supported subgroups. The tree further shows the inclusion of P95 and P151 in the G1 subgroup: P151 clusters with the rabbit samples collected in the same area (P129 and P141-P144), with the Portuguese strains CB137, CB156 and CB194, dating from 1995, 1997 and 2006, respectively, and with the French strain 00–08 dating from 2000; P95 appears to be more closely related to the older European strains belonging to G1, from Spain, France and Germany. The higher average nucleotide and amino acid identity of P95 and P151 is found with G1 (95.9 % - 96.6 % of nucleotide identity and 97.8 % - 98.4 % of amino acid identity). Furthermore, P151 presents 99.8 % - 99.9 % of nucleotide identity and 100 % of amino acid identity with P141- P144.

Figure 4.2 Maximum Likelihood (ML) tree of the capsid gene VP60 for *Lagovirus*. Only bootstrap values ≥ 0.85 are shown. In order to facilitate visualization, major groups are collapsed, with the exception of the group containing the specimens analyzed in the study. The sequences retrieved from GenBank database for this analysis have the following accession numbers: KF494918, KF494921, KF494922, KF494924, KF494930, KF494932, KF494936, KF494943, KF494947, KF494950, KF494951, GU373618, EF558580, M67473, EU650679, EU003579, U49726, AF295785, U54983, FR823355, FJ212323, AF402614, EU003580, EF558579, JN851735, AF231353, DQ189078 (sequences from G2); AM085133, EF558576, EF558572, Y15424, EF558574, AJ535094, AJ535092, Y15426, X87607, FR823354, EF558577, EF558585, Y15441, EF558575, KC595270, AY928268, DQ189077 (sequences from G3-G5); AY523410, KF270630, AF258618, EU003582, DQ205345, DQ280493, AB300693, HM623309, EF558581, EU003578, EU003581, EF558583, EF558582, EF558584, JF412629 (RHDVa sequences); HE800529, FR819781, HE800532, HE800531, HE819400, HE800530, KC345612, JQ929052, KC345611 (RHDV2); Z32526, X98002, U09199, KC832838, KC832839, AM408588, AM933648, AM933649, AM933650, AM887765 (EBHSV sequences). RHDV sequences from Iberian hares appear in bold.

In order to confirm the species identity of P95 and P151, one mitochondrial (cytochrome *b*) and three nuclear markers (*CCR5*, *CXCR4* and CH2 domain of *IGHG*) were amplified for both samples. Total genomic DNA was extracted from the liver samples using the EasySpin Genomic DNA Minipreps Tissue Kit (Citomed, Lisbon, Portugal) according to the manufacturer's instructions. The nuclear markers have been shown to present specific SNP for *Oryctolagus* and *Lepus* and their amplification followed the conditions described in [14-16]. The amplification of the mitochondrial marker was performed using the primers described in [17]. PCR was performed in a final

volume of 10 µL containing 1 µL of DNA, 3 pmol of each primer and 5 µL of Hot-StartTaq DNA Polymerase Multiplex PCR Master Mix (Qiagen) (PCR cycle conditions are available upon request). After purification, PCR products were sequenced with the amplification primers as described above. Sequences of P95 and P151 for all markers were deposited in GenBank under the accession numbers KJ943798-KJ943805. The obtained sequences were then visually inspected and aligned, and compared with publicly available sequences of lagomorphs. Genetic distances between P95 and P151 and the other lagomorph sequences were calculated for each marker in MEGA6 [13], using the p-distance method and 500 replicates; all positions with less than 95 % site coverage were eliminated. Additionally, an ML approach was also used to confirm the phylogenetic position of P95 and P151 in the phylogeny of lagomorphs for each marker, using the best model estimated for each marker (Kimura2 + G for *CCR5* and *CXCR4*; T92 for *IGHG*-CH2 and GTR + I + G for cytochrome *b*) and 500 replicates. The analysis of the genetic distances between P95 and P151 and other lagomorphs indicates that both specimens are closer to *L. granatensis* for all markers (genetic distance of 0.0 % for nuclear genes and 0.6 % for the mitochondrial gene; Table 4.1). Despite the lack of information for some markers, the overall highest genetic distance was observed for *Ochotona* spp: 8.4 % - 18 % for nuclear markers and 19.9 % - 20.3 % for cytochrome *b*. The phylogenetic analysis performed for each marker also supports the clustering of P95 and P151 with *L. granatensis* (data not shown).

3. Discussion

Evolution of RHDV has been widely characterized, with strains grouping according to their year of isolation [10-12,18,19]. In France, strains are divided into six genogroups, G1-G6, and most genogroups have been successively replaced [11]. Interestingly, G1 was able to persist only in the Iberian Peninsula (IP) [10] and, until 2011, it was the only genogroup found, most likely due to the Pyrenees acting as a major natural barrier that limits virus dispersal [10]. The antigenic variant RHDVa was also detected, but only in farms and never in wild populations [20]. From 2011, the new variant RHDV2 or RHDVb was detected and G1 seems to have been replaced [8,21,22]. Interestingly, RHDV2 was recently reported in Cape hares, *L. c. mediterraneus* [6], constituting the first evidence of cross infection in *Lagovirus*. Here we amplified and sequenced the complete capsid protein gene VP60 of a *Lagovirus*, confirmed to be RHDV, from Iberian hares found dead in the field in the 1990s in Portugal.

Table 4.1 Genetic distances of P95 and P151 relative to lagomorphs for nuclear markers *CCR5*, *CXCR4* and *IGHG*-CH2 and mitochondrial marker cytochrome *b*.

	CCR5		***CXCR4***		***IGHG*-CH2**		**cytochrome *b***	
	P95	**P151**	**P95**	**P151**	**P95**	**P151**	**P95**	**P151**
Lepus granatensis	0.0	0.0	0.0	0.0	0.0	0.0	0.6*	0.6*
Lepus europaeus	0.2	0.2	0.5	0.5	0.9	0.9	8.1*	8.1*
Lepus timidus	0.2	0.2	-	-	0.0	0.0	9.7*	9.7*
Lepus americanus	-	-	0.4	0.4	0.9	0.9	9.8*	9.8*
Lepus callotis	2.7	2.7	-	-	1.1	1.1	9.1*	9.1*
Lepus californicus	-	-	0.3	0.3	1.4	1.4	9.7*	9.7*
Lepus townsendii	0.4	0.4	0.3	0.3	-	-	9.4*	9.4*
Lepus capensis	-	-	-	-	1.1	1.1	9.7*	9.7*
Lepus saxatilis	0.0	0.0	0.3	0.3	1.1	1.1	9.8*	9.8*
Lepus castroviejoi	0.4	0.4	0.4	0.4	0.9	0.9	10.9*	10.8*
Sylvilagus brasiliensis	2.3	2.3	1.1	1.1	-	-	-	-
Sylvilagus floridanus	2.9	2.9	0.8	0.8	-	-	15.9*	16.1*
Bunolagus monticularis	5.1	5.1	1.0	1.0	-	-	14.3*	14.5*
Oryctolagus cuniculus	4.9*	4.9*	1.1*	1.1*	4.2*	4.2*	14.7*	14.9*
Ochotona princeps	18.0	18.0	8.9	8.9	-	-	19.9*	20.1*
Ochotona dauurica	-	-	8.4	8.4	-	-	20.2*	20.3*

Only species with information for at least two markers are presented in the table. Values are given in percentages; "-" means lack of information and "*" means average value (obtained by the mean of the genetic distances of three randomly selected sequences for that species).

GenBank accession numbers: DQ146764, DQ146763, GU047870, GU047868, GU047869, GU047871, GU047867, GU047872, DQ017768, GU047865, AM051305, AM051298, DQ017767, GU047873 (*CCR5*); EU258270, EU258265, EU258269, EU258271, EU258266, EU258268, EU258267, EU258273, EU258272, EU258264, EU258287, EU258292, EU258304, EU258275, EU258274 (*CXCR4*); AJ295217, AJ295221, AJ295216, AJ431716, AJ295220, AJ295219, AJ295218, AJ295222, AJ431717, L29172, AY386696, AJ430862 (*IGHG*-CH2); AY942565, HQ596476, AF157465, AY745113, HQ596473, AJ421471, HM233015, AY599076, AB687529, KF781432, KF781431, KF781430, HQ596467, AF010159, HQ596468, KF781354, HQ596464, KF781357, HQ596485, JN037375, JN037374, HM233008, AY292732, EU729261, HQ596480, AF009731, AY292730, AY942569, JN037350, AY176235, AY292724, U58939, AF034257, AY292718, U58931, AY292717, HG810783, HG810780, EU591094, EU591077, EU590965, AF273000, JF911809, EF567059 (cytochrome *b*).

The phylogenetic analysis of the complete capsid gene VP60 revealed that the sequences obtained from the two Iberian hare specimens group with RHDV genogroup 1 (Figure 4.2). Since G1 was the only genogroup known to circulate in IP at the time of sample collection (1996 and 1998), this supports our finding of a cross-species infection of RHDV. Additionally, it appears that two different strains were able to infect hares: the sequences of P95 and P151 present 96 nucleotide differences that translate into 17 amino acid differences in the VP60 (nucleotide genetic distance of 6 % and amino acid genetic distance of 2.8 %; data not shown). Moreover, the strains are located in different nodes of the phylogenetic tree: P151 clusters with a recent French strain and Portuguese strains, including RHDV strains sequenced from rabbits collected in the same location

that also experienced an RHDV outbreak, while P95 is basal to older Spanish, French and German G1 sequences.

Species determination, achieved by the analysis of nuclear and mitochondrial markers, confirmed the hare specimens as being *L. granatensis* (Table 4.1), which is also consistent with the morphological characterization and species geographic range in Portugal [4] (Figure 4.1). Since all five living lagomorph taxa inhabiting the IP (*L. granatensis*, *L. europaeus*, *L. castroviejoi* and both subspecies of *O. cuniculus* – *O. c. cuniculus* and *O. c. algirus*) were represented in these analyses, further confidence is given to the species identification.

L. europaeus is the main host of EBHSV, and *L. timidus* is affected only in regions where *L. europaeus* is also present [2]; no reports of EBHSV exist in *L. granatensis* despite the analysis of high numbers of animals (Gortázar C, personal communication). On the IP, *L. europaeus* is restricted to a region between the Ebro River and the Pyrenees, and a narrow contact zone exists between *L. europaeus* and *L. granatensis* [23] (Figure 4.1). Hence, despite some evidence of ancestral and ongoing hybridization between both species and with *L. timidus* [24], transmission of the virus between these species might be quite limited. In addition, and as for RHDV, the Pyrenees might also restrict EBHSV introduction on the IP. Thus, contact of Iberian hares is more likely to occur with RHDV than with EBHSV.

Our results further show that the RHDV outbreak that occurred at the end of 1997 in a rabbit population, as confirmed by the RHDV positive rabbit samples P141- P144, was the source of the *Lagovirus* that caused the fatal infection in P151. Indeed, P151 was found dead in January-1998, only one month after those rabbits died. The high nucleotide and amino acid identity also confirmed this observation (99.8 % and 100 %, respectively). The possibility that the observed results were due to contamination was discarded given that RNA extraction, cDNA synthesis and PCR amplification of hare samples were performed independently from the rabbit samples. Also, none of the sequences obtained from hares was identical to previously amplified sequences in our facilities or any other sequences available in public databases. Thus, the susceptibility of Iberian hares (*L. granatensis*) to RHDV is confirmed by our results. Nevertheless, how RHDV is able to overcome species boundaries is unknown.

At present, only glycans of the histo-blood group antigens family have been associated with rabbit susceptibility to RHDV [25]; however, preliminary data indicate that glycans are differently located in hare tissues (Le Pendu J, Abrantes J, Lopes AM, personal communication). Other factors could have promoted the cross infection. The

higher mutation capacity of RNA viruses might partially explain their ability to jump species boundaries [26]. Also, the closer the species are to the natural host, the more susceptible they are to the virus [27]. Despite this, the majority of virus transfers to new hosts do not cause severe outbreaks, but only single infections or limited outbreaks [26]. This seems to be the case in our study, where RHDV was able to infect the two hares analyzed, but not capable to cause easily detectable outbreaks. Additionally, viral recombination in unfavorable environments is associated with transmission to new hosts [26]. Although recombination is rare in RNA viruses, there are previous reports of recombination in RHDV [28,29]. Screening of our alignment showed no evidence of recombination; nevertheless, only a quarter of the total viral genome was investigated, so it is unclear if recombination contributed to the host switch. Nonetheless, on two occasions, classical RHDV spilled over into individual Iberian hares. While this is an interesting finding, it has not been reported in Iberian hares since then. Therefore, it is likely that these have been isolated "spillover" events in areas where the habitats of the two hosts overlap and host densities were high [30].

In sum, we report the identification of two different RHDV strains in *L. granatensis* that represent two independent infections, one in 1996 and the other in 1998. This finding of RHDV in Iberian hares represents the earliest evidence of a cross-species transmission of classical RHDV otherwise considered species-specific.

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Leporids tissue binding and glycan specificities of the new RHDV variant and non-pathogenic related viruses

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1. Abstract

The rabbit hemorrhagic disease virus (RHDV) and the European brown hare syndrome virus (EBHSV) are two lagoviruses from the family *Caliciviridae* that cause highly fatal diseases in two leporid genera, *Oryctolagus* and *Lepus*, respectively. In the last few years, several examples of host jumps of lagoviruses among leporids were described. The causes for these host shifts are unknown, but they are more likely among closely related species. Recombination is also frequently associated with the ability of viruses to establish in a new host and has been described for RHDV. Host jumps allow viruses to expand their host range and are far more common in single-stranded RNA viruses, which include RHDV and EBHSV. Understanding how and why viruses cross the species boundaries is important to predict future cases. Here, we studied the interaction between the pathogenic new RHDV variant and non-pathogenic lagoviruses from Europe and Australia with three lagomorph genera (*Oryctolagus*, *Lepus* and *Sylvilagus*). Binding of each virus to the upper respiratory and digestive tracts was similar to that observed for the classical RHDV. The glycans to which viruses attach to were compared and histo-blood group antigens (HBGAs) were the most common attachment factors, along with heparin. However, the binding pattern of each virus to HBGAs was variable and might be related with adaptation to host evolution.

2. Introduction

The high mutation rates, vast population sizes and short generation times make RNA viruses capable of rapidly adapt to a large number of hosts and thus prone to cross species boundaries [1]. Viruses are more likely to jump between closely related species

[2,3], which may result in unique spillover infections or severe epidemics, depending on how successfully the virus adapts to the new host population. Host switching constitutes an important mechanism on virus evolution [4,5] and is important in several families of viruses, including *Caliciviridae*. Caliciviruses comprise five recognized genera: *Norovirus*, *Sapovirus*, *Vesivirus*, *Nebovirus* and *Lagovirus* [6]. The genus *Lagovirus* encompasses two species: rabbit hemorrhagic disease virus (RHDV), highly fatal to the European rabbit (*Oryctolagus cuniculus*), and European brown hare syndrome virus (EBHSV), that affects European brown and mountain hares (*Lepus europaeus* and *L. timidus*, respectively). These two non-enveloped viruses have a single-stranded, positive-sense RNA genome [reviewed in 7].

Both RHDV and EBHSV emerged in the 1980s [8,9] and are similar in terms of morphology, genome organization and epidemiological course of the respective diseases, rabbit hemorrhagic disease (RHD) and European brown hare syndrome (EBHS) [10,11]. For EBHSV, only a single serotype is known [12] and no cases of non-pathogenic forms of the virus circulating in hare populations are described. In contrast, several genogroups are recognized for RHDV [13], including the antigenic variant RHDVa [14], and several non-pathogenic and moderately pathogenic strains also circulate in rabbit populations from all over the world [15-18]. In 2010, a new pathogenic RHDV variant emerged and rapidly spread throughout Europe and Australia [19-26].

Virus attachment to the cells of the new host constitutes a crucial step for a species jump and will determine virus entry. Attachment factors include proteins, carbohydrates and lipids. Regarding caliciviruses, the most common ligands are carbohydrates: murine norovirus and feline caliciviruses use sialic acid [27,28], human norovirus recognizes heparan sulfate [29], histo-blood group antigens (HBGAs) are used by noroviruses and RHDV [30-33], and EBHSV is thought to bind to GlcNAc-terminated O-glycans (Lopes *et al.*, unpublished results).

In Europe, three genera of lagomorphs exist: *Oryctolagus* (rabbits), *Lepus* (hares) and *Sylvilagus* (cottontails), which recently diverged (~ 12 million years ago) [34]. *Sylvilagus* is a native American genus, but has been successively introduced in Europe in the 1960s and 1970s [35]. In Italy, it has adapted to the new environment and may have caused a decline in hare populations [36]. Six hare species are dispersed throughout Europe, with *L. granatensis* and *L. castroviejoi* in the Iberian Peninsula, *L. timidus* in the northern Europe, *L. capensis* in Sardinia, *L. corsicanus* in several regions of Italy, and *L. europaeus* widespread in central Europe [37]. *Oryctolagus cuniculus* is distributed all over Europe and may occur in sympatry with hares and cottontails.

RHDV and EBHSV experimental cross-infections in hares and rabbits have been attempted, but results were quite disparate with some studies failing to induce disease [e.g. 38,39,40], while others reported successful cross-infection [41,42]. Additionally, cottontail challenges with EBHSV resulted in infection and death of one animal [43]. More recently, several cases of cross-species infection occurring in natural conditions were reported: Iberian hare (*L. granatensis*) with RHDV infection [44]; eastern cottontail (*Sylvilagus floridanus*) susceptible to EBHSV [45]; and *L. capensis* and *L. corsicanus* were found to be fatally susceptible to the new RHDV variant [46,47] (see Table 4.2 for a summary of the available information).

Table 4.2 Reported cases of cross-transmission in lagomorph species inhabiting Europe. Only pathogenic viruses are considered.

	RHDV	**RHDV2**	**EBHSV**
O. cuniculus	Natural host [8]	Natural host [19]	No reports
L. europaeus	No reports	Field evidence*	Natural host [9]
L. timidus	No reports	No reports	Natural host [9]
L. granatensis	Field evidence [44]	No reports	No reports
L. corsicanus	No reports	Field evidence [46]	Field evidence [48]
L. capensis	No reports	Field evidence [47]	No reports
L. castroviejoi	No reports	No reports	No reports
S. floridanus	No reports	No reports	Field and experimental evidence [43,45]

* Le Gall-Reculé *et al.*, data not published

Two hypotheses for the emergence of pathogenicity in lagoviruses are currently accepted. The first refers to the emergence of virulence from non-pathogenic circulating viruses that, by reasons not directly related with the host, acquired pathogenicity. This hypothesis is supported by the detection of antibodies against RHDV and EBHSV in samples collected before virus emergence [e.g. 49,50,51] and by the characterization of non-pathogenic forms of the rabbit caliciviruses [e.g. 15-17,18,52]. The other hypothesis involves a species jump, most likely from *Sylvilagus* [53], where viruses would circulate as benign forms. This is consistent with the dates of introduction of *Sylvilagus floridanus* in Europe and subsequent emergence of RHD and EBHS [8,9,54,55].

We investigated the ability of the new RHDV variant and non-pathogenic lagoviruses from Europe and Australia to bind to leporids other than their natural hosts and the required host attachment factors to start infection. The close phylogenetic relationship of these leporids [34], together with an overlapping distribution [37] and an

overall conservation of glycans among vertebrates, prompted us to consider them as good models to test the cross-species hypothesis.

3. Methods

Sample collection

Scrapings of rabbit duodenum were prepared from wild rabbits killed by hunters in the French Pyrenees and previously phenotyped for HBGAs [30]. Tissue samples of four *L. europaeus* and three *L. granatensis* were collected as part of a previous study (Lopes *et al.*, unpublished results); tissue samples of *O. cuniculus* were obtained from 12 laboratory animals and eight wild animals from France, and six *S. floridanus* samples were collected in a farm in southern France. Duodenum and trachea samples were either collected in 10 % buffered formalin for immunohistochemistry studies or in RNA later for ELISA. Tissue sections and scrapings were prepared as described in [30] and HBGAs phenotyped accordingly.

Virus samples were obtained from liver homogenates (10 % (w/v) in phosphate buffered saline, PBS) of dead animals.

Preparation of VLPs

Virus-like particles (VLPs) were constructed for the European non-pathogenic caliciviruses strains 06-11 [17] and RCV-E1 (Le Gall-Reculé, unpublished observations) and for the new RHDV variant strain 10-28 [20]. VLPs were generated according to [56]. The Australian rabbit calicivirus (strain RCV-A1) VLP was previously prepared for another study [57]. Briefly, the complete capsid protein gene sequence was determined by PCR and sequenced for each strain; recombinant baculoviruses expressing the VLPs were generated and Sf9 cells infected. Cellular debris and baculovirus were removed by centrifugation (10,000 rpm, 30 minutes) and freeze-thawing cycles released VLPs from the cells. The supernatant was once again centrifuged at 27,000 rpm for 3h and pellets were resuspended in 200 µL of PBS. A caesium chloride solution (0.4 g/mL) was added to the preparation and ultracentrifuged for 18h at 36,000 rpm. VLPs' fraction was collected by puncture and dialyzed against PBS. Caesium chloride was eliminated through serial washes in Vivaspin columns 30000 MWCO PES. The integrity and quality of the VLPs was checked by Coomassie blue staining of SDS-page gels and Western

blot. Protein amounts were quantified using a Nanodrop 2000 apparatus (Thermo Scientific).

Production of hyperimmune antiserum

A hyperimmune serum that recognizes the new RHDV variant and the rabbit caliciviruses 06-11 and RCV-E1 was produced. For this, two rats were serially inoculated with the 10-28 VLP. A monoclonal antibody (MAb 11F12) that recognizes the Australian rabbit calicivirus RCV-A1 was previously prepared [57]. Rat inoculation was performed at the animal experimentation core facility of the University of Nantes and was approved by the national ethic review board from the French Ministry of Enseignement Supérieur et de la Recherche (project license number 01322.01). Animal care and use protocol adhered to the European Directive number 2010/063 and to the national French regulation (Décret no. 2013-118 du 1er février relatif à la protection des animaux utilisés à des fins scientifiques). The antibody generated was tested by ELISA for the recognition of target VLPs.

Immunohistochemistry and ELISA virus binding assays

Tissue sections of *O. cuniculus*, *L. granatensis*, *L. europaeus* and *S. floridanus* were deparaffinized with LMR-SOL baths and rehydrated with successive ethanol baths. Hydrogen peroxide 0.3 % (v/v) in phosphate buffered saline (PBS) was added to the slides to block endogenous peroxidase activity, followed by bovine serum albumin (BSA) 3 % (w/v) in PBS to block non-specific binding. VLPs at 10 µg/mL were incubated with the tissue sections at 4 ºC overnight. Monoclonal antibodies or hyperimmune sera (see Table 4.3) were then incubated with the tissue sections for one hour at 37 ºC, followed by a biotinylated or HRP-conjugated secondary antibody for one hour at 37 ºC. For those tissues incubated with the biotinylated antibody, a last incubation with HRP-conjugated avidin D was performed, for one hour at room temperature. In between incubations, slides were washed three times with PBS for 5 min. Substrate (AEC kit) was added to the slides and Mayer's hematoxylin was used for contrast staining.

Tissue scrapings of *O. cuniculus*, *S. floridanus*, *L. europaeus* and *L. granatensis* were coated in Nunc Maxisorp plates in 2-fold dilutions, starting at 10^{-2}. Sodium carbonate buffer, pH 9.5, at 100 mM was used as coating buffer and samples were incubated overnight at 4 ºC. Blocking of the plates with 5 % non-fat dry milk diluted in distilled water or PBS was followed by the VLPs at 8 or 12 µg/mL. Monoclonal antibodies

or hyperimmune antisera used for each virus are indicated in Table 4.3. TMB substrate was used to reveal and 1 M phosphoric acid stopped the reaction. Optical density (O.D.) was measured at 450 nm. All incubations were at 37 ºC in humid atmosphere; samples and antibodies were diluted in PBS or distilled water according to the solvent of the blocking solution; plates were washed three times with 0.05 % Tween 20 in PBS after each incubation.

Table 4.3 Antibodies and conditions used for IHC and ELISA binding of viruses to *Oryctolagus*, *Sylvilagus* and *Lepus*.

VLP	Primary antibody	Secondary antibody
10-28	α10-28	α-rat HRP*
06-11	α10-28 / SD4	α-rat HRP*
RCV-E1	α10-28	α-rat HRP‡
RCV-A1	11F12	α-mouse HRP#

*A biotinylated secondary antibody was used and avidin D-HRP was added in IHC for *Lepus* binding.

‡A biotinylated secondary antibody was used and avidin D-HRP was added in IHC for *Sylvilagus* and *Lepus* binding.

#A biotinylated secondary antibody was used and avidin D-HRP was added in IHC for all genera.

ABH phenotype-dependent binding

The attachment ability of each virus for animals with different ABH phenotypes (A^+B^+, A^+B^- and A^-B^-) was tested by ELISA; rabbit samples representative of each phenotype were used to this end. Three to five samples of each phenotype were selected and the binding of each virus tested. ELISA protocol was performed as for the virus binding to the tissues referred above.

Printed glycan arrays (PGAs)

Printed glycan array slides were manufactured and profiled as described in [58]. Briefly, six replicates of 360 monomeric glycans (50 µM) as ω-aminopropyl glycosides of 95-98 % purity were diluted in 300 mM PBS/0.005 % Tween 20 (pH 8.5) and printed by robotic pin deposition on *N*-hydroxysuccinimide activated glass slides. Free *N*-hydroxysuccinimide activated groups were blocked with 50 mM ethanolamine in 50 mM borate buffer at a final pH of 9.2. Slides were then rinsed with deionized water, dried and stored at room temperature in a dessicator. Each VLP was incubated on the slides diluted PBS-Tween BSA (0.1 % (v/v) Tween 20 and 3 % (w/v) BSA) and incubated in humid chamber for 2h at 37 ºC with gentle shaking. Monoclonal antibodies or

hyperimmune sera also diluted in PBS-Tween BSA (see Table 4.3) were incubated at room temperature for 45 minutes. A final incubation with Cy5-labeled secondary antibodies diluted in PBS-Tween (0.1 % (v/v) Tween 20) was performed at room temperature for 30 minutes. In between incubations, slides were washed with a series of 0.1 % and 0.001 % Tween 20 in PBS. Fluorescence signals were measured with an Agilent scanner G2565CA and analyzed using ImaGene analysis software version 7.5 (BioDiscovery, El Segundo, USA).

Virus binding to synthetic sugars

The binding of each virus to selected synthetic sugars, based on the classical RHDV binding patterns [30,31], was evaluated. A panel of PAA-conjugated sugars were coated at 10 µg/mL and incubated overnight at 4 ºC. The plate was blocked with 5 % non-fat milk/H_2O for one hour at 37 ºC and then incubated with the VLPs at 10 µg/mL. The specific antibodies for each virus, as indicated in Table 4.3, were then used as detailed above, as well as the remaining ELISA conditions.

4. Results and Discussion

RNA viruses are prone to cross the species boundaries, probably due to their high mutation rates, short generation times and large population sizes that enable them to quickly adapt to new hosts [1,59]. In addition, analysis of past host shifts accentuates single-stranded RNA viruses as being often involved in species jumps [60-62]. Phylogenetically related species are more likely to experience species jumps as they have more similarity in the cell types, receptors and other cell components key to the viral replication [2,5,63,64]. Recombination is also likely to influence the epidemiology of species jumps by allowing different genome combinations to be “tested” in the new host [65]. While in most cases it is not possible to directly relate recombination with host jumps, several examples are found [e.g. 66,67,68].

Caliciviruses are good models to study species jumps. Indeed, caliciviruses are single-stranded RNA viruses, recombination was detected in members of all genera of this family [69-77], and several indications of the possibility of host jumps have recently been described [78,79]. For *Lagovirus*, spillover infections have been reported, including the classical RHDV in *L. granatensis* [44], the new RHDV variant in *L. corsicanus* and *L. capensis* [46,47], and EBHSV in *S. floridanus* [43,45].

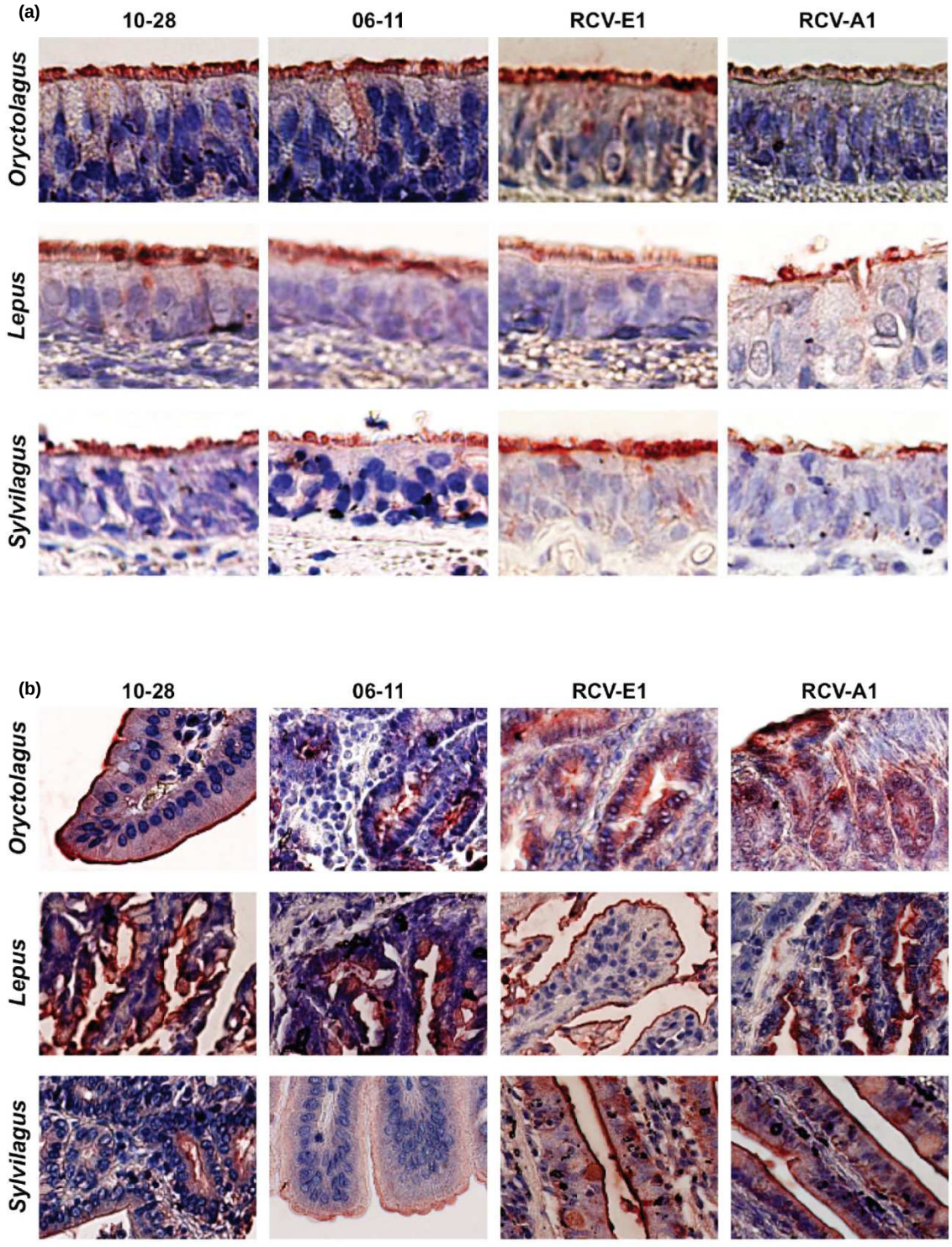


Figure 4.3 Binding to leporids' trachea (a) and duodenum (b) of lagoviruses. All viruses bind to the epithelial layer of trachea (a) and to the Lieberkühn crypts (b). Controls performed in the absence of VLPs presented no red/brown staining (not shown). 10-28: new RHDV variant; 06-11 and RCV-E1: European non-pathogenic caliciviruses; RCV-A1: Australian non-pathogenic calicivirus.

Our immunohistochemistry results showed that, similar to the classical RHDV, the viruses tested were able to bind to the epithelial cells of both the upper respiratory and the digestive tract of all the analyzed genera, and no obvious differential binding was observed (Fig. 4.3a and 4.3b). These results suggest that all leporids present appropriate attachment factors on their epithelial cells that might allow lagoviruses to initiate infection even in non-natural hosts. When comparing the attachment ability of these viruses to animals of different ABH phenotypes, variations in attachment preferences were observed. Indeed, the non-pathogenic 06-11, RCV-E1 and RCV-A1 showed an ABH-phenotype dependent binding to rabbit tissues similar to that previously observed for some classical strains [30], with a rank of preferences from the A^+B^+ phenotype to A^+B^- and to A^-B^- (Fig. 4.4).

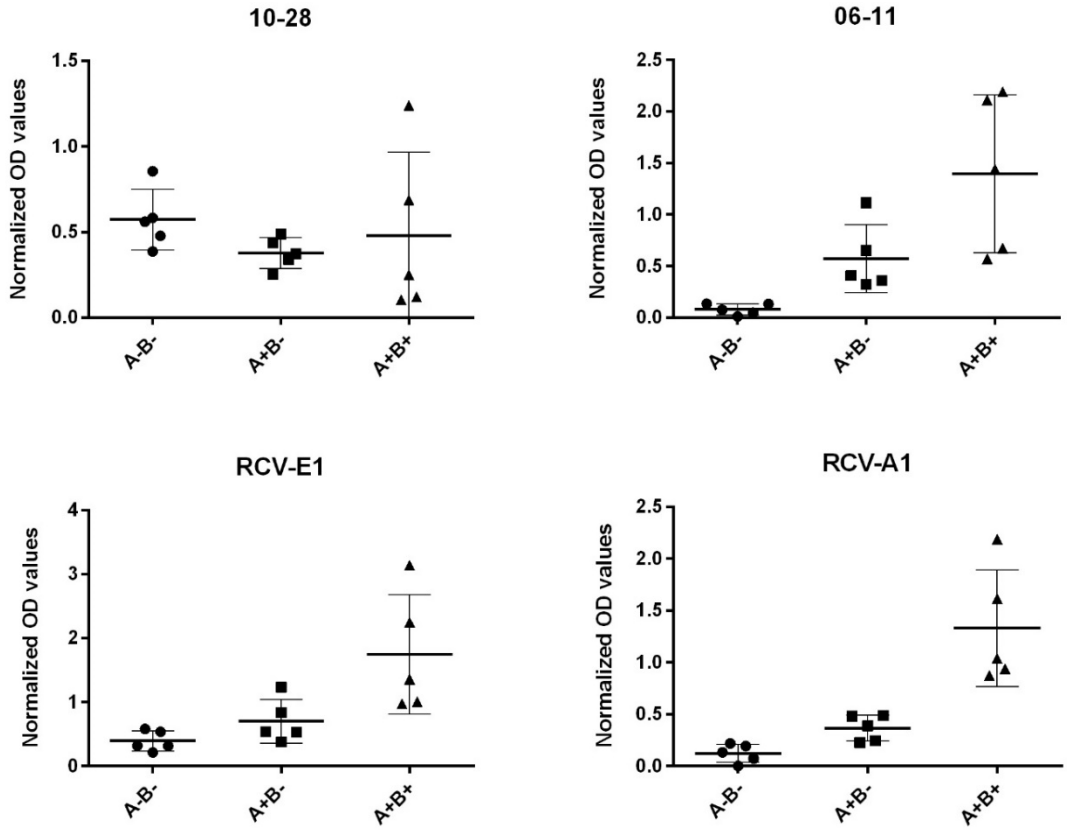


Figure 4.4 ABH phenotype-dependent binding of the new RHDV variant (10-28) and the non-pathogenic strains (06-11, RCV-E1 and RCV-A1). Circles, squares and triangles represent the normalized OD value for rabbit individuals with the ABH phenotype identified on the x axis. Horizontal bars indicate median values for each group.

These results show that both (classical) pathogenic and non-pathogenic forms present the same binding preference for the B antigen, indicating that binding to this motif is not involved in virulence [30]. Interestingly, in hares, the B antigen is absent (Lopes *et al.*, unpublished results), which likely led EBHSV to find an alternative attachment factor. Preliminary results showed that EBHSV preferentially uses N-terminal GlcNAc residues and heparin instead of HBGAs as attachment factors (Lopes *et al.*, unpublished results). The new RHDV variant also showed no ABH phenotype preference (Fig. 4.4), which might suggest the use of alternative glycans, or a lesser dependence on the B blood group antigen. These alternative glycans probably facilitated the infection of new hosts. Indeed, hare species have been shown to be infected with the new RHDV variant [46,47], and maintenance of the B antigen as main attachment factor would have compromised the ability of the new variant to infect these species.

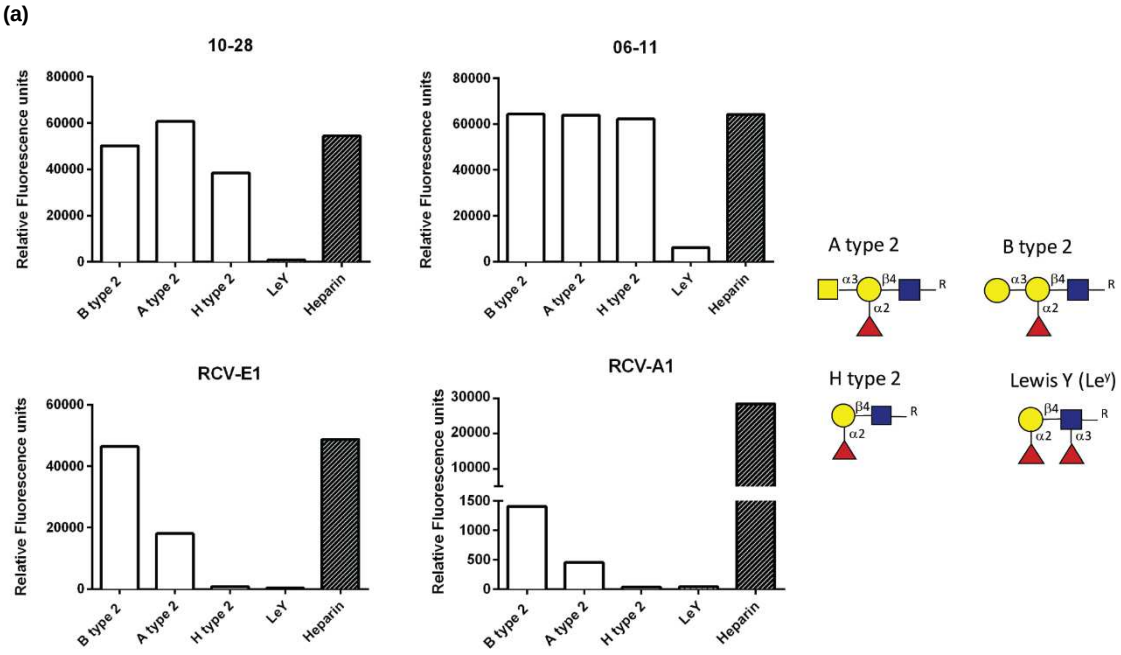
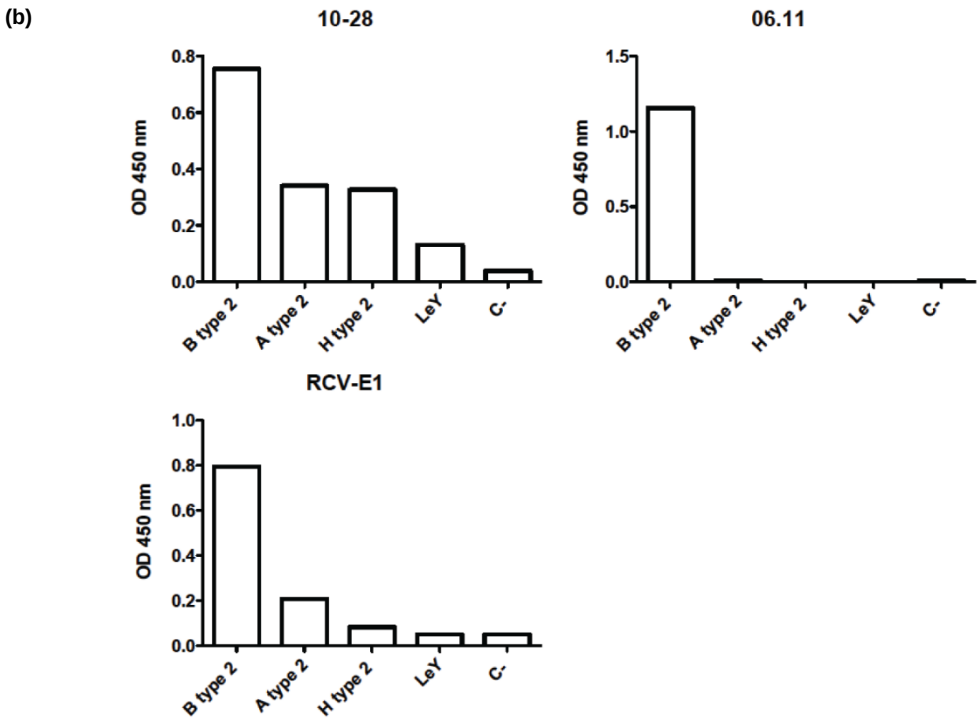


Figure 4.5 Variation in attachment preferences of these viruses. (a) Printed glycan arrays results for the HBGA related glycans and for heparin. The structures corresponding to A type 2, B type 2, H type 2 and Ley are shown on the right, with blue squares representing N-acetylglucosamine, yellow circles representing galactose, red triangles representing fucose and yellow square representing *N*-acetylgalactosamine. **(b)** Binding of VLPs from the new RHDV variant (10-28) and the two non-pathogenic strains (06-11 and RCV-E1) to the HBGAs oligosaccharides conjugated to polyacrylamide by ELISA. In these conditions no binding was observed for the RCV-A1 strain (not shown). C- shows OD values obtained in the absence of polyacrylamide conjugate.

Figure 4.5 (cont.)



In order to improve our understanding of the viruses glycan-binding properties, printed glycan arrays were used. These arrays allow to assay simultaneously a large number of glycan structures. The main finding is that all lagoviruses, but EBHSV (Lopes *et al.*, unpublished results), bind to histo-blood group antigens (HBGAs) with all the analyzed rabbit lagoviruses binding to B type 2 (Fig. 4.5a), which was established as the top binder for classical RHDV [30,31]. Binding to either A type 2 and H type 2 was also observed but was lower than to B type 2 for the non-pathogenic strains RCV-E1 and RCV-A1, consistent with their preferential recognition of A^+B^+ rabbits over A^+B^- rabbits and their very low binding to tissue extracts of A^-B^- animals that only possess the H type 2 epitope [30]. Strong binding of the new pathogenic variant strain (10-28) to either B type 2, A type 2 and H type 2 explains the lack of preference for animals of a particular ABH phenotype. Indeed, A^+B^+ animals strongly express both the A and B type 2 epitopes that are well recognized and only small amounts of H type 2 which is masked by the A and B epitopes. Animals of the A^+B^- phenotype express strong amounts of the A type 2 epitope, but no B type 2 and only small amounts of H type 2, whilst the A^-B^- animals express only H type 2 albeit in large amounts since it is not masked by the presence of the A and B epitopes. Thus, the broad specificity of the new variant for A, B and H type 2 explains its broad recognition of rabbits regardless of ABH phenotypes.

For the 06-11 non-pathogenic strain, an apparent discrepancy between the printed glycan array data and the ELISA data on rabbit duodenal scrappings was observed since that strain showed a clear preference for animals of the $A^+B^+ > A^+B^- > A^-B^-$ phenotypes, whilst no preference for the B type 2 epitope over either the A type 2 or the H type 2 epitopes was noticed. This might be explained by the array reading that was mistakenly performed at saturation so that no quantitative difference could be made between the positive glycan structures. To check for that possibility and to confirm results obtained for the other strains, the A, B and H type 2 oligosaccharides conjugated to polyacrylamide (PAA-conjugates) were immobilized on plates and VLPs binding was assessed by ELISA (Fig. 4.5b). This confirmed that the new pathogenic variant recognized the three motifs A type 2, B type 2 and H type 2, whilst the RCV-E1 non-pathogenic strain showed a clear preference for B type 2 over A type 2 and lacked recognition of H type 2. As to the 06-11 strain, it showed binding to B type 2-PAA exclusively. No binding to A type 2 was detected in these conditions, indicating that its recognition on the printed glycan array reflected a weak binding detected due to the very high sensitivity of the system. Overall these data showed a remarkable consistency between recognition of synthetic HBGA motifs and binding to subgroups of rabbits defined by their expression of ABH epitopes.

In addition to HBGAs, the printed glycan arrays revealed strong binding of all VLPs to heparin which represents a distinct type of glycan (Fig. 4.5a). It also revealed binding to several other sulfated glycan motifs (data not shown). Interestingly, all known lagoviruses, including EBHSV and the classical RHDV (data not shown), bind to heparin. Heparin is a highly sulfated glycosaminoglycan chemically related to heparan sulfate, a glycan ubiquitously expressed in virtually all cell types and used by many viruses to attach to host cells, including other caliciviruses [29,80-84]. In addition, the other glycans used by EBHSV as attachment factors are remarkably different from the other lagoviruses (Lopes *et al.*, unpublished results). This might relate to the fact that EBHSV was never reported in the European rabbit. The comparison of the binding of each strain with HBGA-related and non HBGA-related glycans revealed different patterns. While the European non-pathogenic strains (06-11 and RCV-E1) have a preference for HBGA-related glycans, the Australian non-pathogenic strain presents a preference for glycans non-HBGA related as can be seen from the very high binding to heparin relative to B type 2 (Fig. 4.5a) and the lack of binding to HBGA as PAA-conjugates (data not shown).

Both pathogenic and non-pathogenic strains were able to recognize and bind to attachment factors present in the different leporids studied, including *Sylvilagus* (Fig. 4.3). This suggests that all leporids are susceptible to both pathogenic and non-

pathogenic strains, at least at the very first step of the virus life cycle, since the attachment factors seem to be shared between species, which might facilitate cross-infection. However, some glycan preferences were observed indicating somewhat different binding requirements for the different lagoviruses which could explain the lack of reports of some cross-species infections (e.g. RHDV in *Sylvilagus*). Furthermore, when adapting to a novel host, virus fitness on the original host might be reduced [4]. This might explain why *Sylvilagus* is experimentally susceptible to EBHSV and not to RHDV [45] although no apparent barriers exist in the eastern cottontail that impair RHDV binding, since it is capable of attaching to the epithelial cells of *Sylvilagus*. Thus, factors other than the attachment might account for the lack of success of RHDV in *Sylvilagus*.

The new RHDV variant has been remarkably successful in infecting species other than the natural host, since it was able to infect several *Lepus* species: *L. capensis* [47], *L. corsicanus* [46] and *L. europaeus* (Le Gall-Reculé *et al.*, unpublished results). This is consistent with its broad HBGA specificity. HBGA expression of *Sylvilagus* is similar to that of *Oryctolagus*, and although hares do not express the B type 2 epitope, which is the best ligand for the classical RHDV and the non-pathogenic strains, the ability of the new variant to strongly recognize A type 2 and H type 2 that are expressed by hares might contribute to the reported cross-infection events with that strain. Alternatively, the new RHDV variant might have acquired the ability to bind to several attachment factors, including non-HBGA related glycans. Whether recombination had a role in crossing the species barrier remains to be determined, but the high rate of recombination [77] and the maintenance of recombinant and non-recombinant forms in wild rabbit populations suggest some adaptive advantage. Interestingly, all these reports of species jumps occurred in areas where these *Lepus* species live in sympatry with *O. cuniculus*. This might explain the absence of reports of the new RHDV variant on *L. timidus* as this species' distribution only slightly overlaps *O. cuniculus* distribution. However, no cases of the new RHDV variant have been reported in Iberian *Lepus* species (*L. granatensis* and *L. castroviejoi*) despite an overlapping distribution with *O. cuniculus*. The case of *L. granatensis* is particularly interesting, since there is a report of two hares that died from RHD caused by an infection with classical RHDV [44], but no widespread infection was detected, suggesting this was a dead-end spillover infection. In addition, hundreds of Iberian hares were tested for EBHSV and no cases were detected (C. Gortázar, personal communication). This is likely because *L. europaeus* and *L. granatensis* have only a narrow contact zone near the Pyrenees hindering transmission of EBHSV [37].

Whether *Lagovirus* attachment factors are determinant for successful species jump is unknown, but it is clear that they play a role in RHDV infection [30,85,86]. Our results

show that the leporids studied have, at least at the first step of the virus life cycle in the host, the attachment factors required for both pathogenic and non-pathogenic strains to initiate infection. However, lagoviruses species jumps seem to be limited by other factors that might include overlapping distribution, viral loads and population density, but also the existence of appropriate receptors or the existence of species-specific anti-viral factors. For example, a novel mechanism of RHDV resistance involving the neofunctionalization of Sec1 was suggested [85]. Interestingly, Sec1 is a pseudogene in several mammals but in leporids is involved in an extensive gene conversion with Fut1 and Fut2 [85,87].

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Chapter 5

Final considerations

1. **General discussion**
2. **Future perspectives**
3. **References**

1. General discussion

The new RHDV variant in Iberian Peninsula: major consequences and implications at the molecular level

A major goal of this dissertation was to contribute to the characterization of the new RHDV variant, tentatively named RHDV2 or RHDVb. After its emergence in France in 2010 [1], the new variant spread to Spain, Italy, Germany, United Kingdom and, more recently, to Australia [2-6]. In Portugal, RHDV2 is present in wild rabbit populations since 2012, and occurs all over the country, with special incidence in the south, where wild rabbits are abundant. Both *Oryctolagus cuniculus cuniculus* and *O. c. algirus* populations are affected. Prior to 2012, only G1 was circulating in Iberian wild rabbit populations [7-10] and a few cases of G6 were detected in rabbitries [9,11].

A scenario of replacement of G1 by RHDV2 in Portugal was observed, since G1 was no longer detected after the emergence of RHDV2 [7-10,12,13]. The high dispersal ability of RHDV2 [14] and the replacement of G1 suggest a selective advantage of the new variant. The replacement of older strains by RHDV2 was also described in other countries [2,14,15]. This phenomenon of replacement of older strains by new strains that present a selective advantage is described for several viruses [16,17], including human norovirus [18]. These strains are usually antigenically different and escape the host herd immunity, a scenario that might be compatible with the one observed for RHDV2. The replacement of strains has also been previously described for RHDVa [19,20] and for classical RHDV in France [21].

Whether the emergence of RHDV2 had an impact on the host immune system and its repertoire, as a result of the co-evolution between host and pathogen, is uncertain at the moment. However, in light of the Red Queen hypothesis, it is expected that the high virulence of RHDV2 will increase selection for resistant hosts. Thus, in a few years, resistant/surviving rabbits will start to appear and increase in frequency and the cyclical process of adaptive genetic change will proceed [22]. This is consistent with the increased selective pressure of RHDV after the first epidemic [7]. However, even in the presence of strong selective pressures, several generations will likely be needed to detect the molecular signatures of the genetic conflict in the host. This asymmetry in the

arms race results from the longer generation times of the host compared to the pathogen, along with the lower evolution rates and smaller population sizes [23].

Recombination and its role in the emergence of virulence in the new RHDV variant

In this last five years, several authors have worked on the description of the new variant, including on the associated pathology and the characterization of the virus [e.g. 2,24,25]. One of the most striking findings of this thesis was the report of several recombination events in RHDV2 genomes from the Iberian Peninsula. Recombination is associated with the emergence and increases in virulence of several viruses [26-31].

Why recombination occurred in the new RHDV variant is difficult to assess, but it might be linked to its emergence. Molecular evidence suggest that the RHDV strain from the first highly lethal outbreak in China in 1984 [32] is the product of a recombination event [33]. Nevertheless, not all pathogenic forms are directly descendants of this first strain [34] and recombination is not widespread in all RHDV lineages [35], although this might be due to non-systematic sampling or the lack of full genome sequences for older strains. Other recombination events scattered throughout the genome were detected for the classical RHDV [36,37].

Recombination might also be at the origin of RHDV2. The factors underlying pathogenicity and virulence of RHDV2 are not clear, but mutations in the non-structural protein p16 were recently associated with virulence of RHDV [38]. However, inferring the origin of RNA viruses is extremely difficult [39]. Recombination may have assisted the spread of RHDV2 by helping the virus to acquire mutations more rapidly than mutation alone [40]. Moreover, if we consider that recombination played a role in the origin of RHDV2, leaving the door open for the possibility of other European strains actually being recombinants, the increased virulence of RHDV2 and its high capacity of host jump (that will be analyzed in more detail further on) might be related to recombination [reviewed in 41,42].

The specific pattern of recombination with a consistent recombination breakpoint between non-structural and structural proteins is likely to produce viable recombinants [43] and was reported for other caliciviruses [44], but never for lagoviruses. Nonetheless, when recombining strains are very similar, these events remain undetected [45]. Thus, recombination might have occurred frequently in the past history of RHDV, but the lack

of full-genome sequences and the similarity between strains impaired its detection. Furthermore, the homologous recombination occurring in RHDV2 implies that co-circulation of the strains that gave rise to the recombinants must have occurred, i.e., co-circulation of G1 and RHDV2 and co-circulation of a non-pathogenic strain and RHDV2. The former event was detected only once in a Spanish farm [14] whilst the later was undetectable since non-pathogenic strains have not yet been described in the Iberian Peninsula.

The new RHDV variant in the islands and its impact in wild rabbit populations

Outbreaks caused by RHDV2 were also reported in wild and domestic rabbits of the Portuguese archipelago of Azores [46-48]. Rabbits were introduced in Azores by Portuguese settlers in the 15th century [49] and belong to the subspecies *O. c. algirus*. RHD occurs in these islands since the late 1980s, but until recently little was known on which RHDV strains were circulating in the archipelago. The genomic analysis revealed the presence of G3-G5 *like* strains, with more than 17 years of independent evolution [9,50]. After the arrival of RHDV2, no more cases of G3-G5 *like* strains were observed.

The origin of Azorean RHDV2 strains is likely the Iberian Peninsula, since the genomic characterization revealed that all Azorean strains are G1/RHDV2 recombinants. Indeed, G1 only persisted in Iberian Peninsula [7-10] and, more importantly, G1/RHDV2 recombinants were described in Portugal in the beginning of 2014. In the archipelago, the first cases of RHDV2 date back to November 2014, and in a few months RHDV2 was present in eight out of the nine islands (no wild rabbits being present on the last island, Corvo). In contrast, the arrival of the G3-G5 *like* may have had an origin outside of Iberian Peninsula, given the characteristics of the strains that circulated in Azores and the absence of reports of such strains in the Iberian Peninsula.

The remarkable spread of RHDV2 was certainly helped by anthropogenic factors, in at least two different moments: (i) in the introduction of RHDV2 in Azores itself, since the archipelago is geographically isolated (~ 1500 km apart from mainland Portugal) and thus translocation of rabbits for hunting purposes or contaminated clothes, for example, are the most likely sources of the virus; and (ii) in the dispersal between islands, since transmission by vectors such as insects and birds is not compatible with the dispersal routes, and with the Azorean fauna [51]. Moreover, rabbits from Flores had never been in contact with the disease, indicating that RHDV did not arrive to that island by natural

vector transmission (P. J. Esteves, personal communication). Surprisingly, the RHDV2 strain that arrived to Australia in May 2015 is a G1/RHDV2 recombinant, and the analysis of the genome indicates that it likely originated in southern Europe [6]. At the moment, this seems to be the most widespread of the described RHDV2 recombinant forms.

The new RHDV variant seems to have changed the epidemiology of RHD. The devastating effects on wild rabbit populations from Azores determined the prohibition of hunting in all islands in March 2015. In the Iberian Peninsula, the new RHDV variant has also been decimating wild rabbit populations [52], probably not as dramatically as the first RHDV outbreaks due to the partial cross-protection between RHDV and RHDV2 [2]. In a cascade effect, endangered predators highly dependent on wild rabbits, such as the Iberian lynx (*Lynx pardinus*), had also been affected [52]. In Portugal, control programs were implemented to monitor the status of wild rabbit populations. Unfortunately, vaccination is unfeasible and ineffective in wild rabbits [53]. Regarding domestic rabbits, a new vaccine has already been implemented in France and Spain to control outbreaks in farms [54]. However, as rabbit kittens are also affected by the new variant, a revision of the vaccination protocol should be performed to ensure more adequate vaccination programs.

Insights into the evolution of EBHSV

Our first approach of sequencing the whole genome of EBHSV samples from the first outbreaks in the area of original diagnosis, Sweden, intended to provide information for a deeper virus characterization. Comparing to RHDV, the number of full-genome sequences publicly available is remarkably lower (*ca* 100 for RHDV *vs.* three for EBHSV; data accessed in January 2016). Nonetheless, we confirmed that EBHSV and RHDV genomes have a similar structural organization, as previously reported by other authors [55,56].

The availability and analysis of whole genome sequences of viruses might shed light on fundamental aspects of their biology, but might also benefit vaccine design and vaccination strategies by giving further insights into transmission traits, viral evolution and its association with pathogenesis [57,58]. In light of the recent findings for RHDV, the importance of analyzing full-genome sequences instead of partial portions of the genome is confirmed by (i) the occurrence of recombination outside of the RHDV VP60 [13,33] and (ii) the association of mutations in the non-structural protein p16 and higher virulent RHDV strains from Australia [38].

The characterization of the molecular evolution and antigenic variation of EBHSV further provided insights into the characterization of this virus. The use of sequences from the area of original diagnosis was a significant step in understanding EBHSV molecular evolution. The sequencing of EBHSV samples from European brown hares (*L. europaeus*) and mountain hares (*L. timidus*) also supported that occurrence of EBHS in mountain hares is only residual [59] and no significant differences exist in the genome of strains circulating in these species. European brown hares and mountain hares occur in different habitats, with the former living predominantly in agricultural fields and the latter inhabiting forests. Therefore, contact between them is limited to the margins of forest areas, which reduces the likelihood of occurrence of EBHS outbreaks in mountain hares [59].

No recombination events were yet detected for EBHSV that might have assisted the origin of pathogenicity, contrarily to what happens for RHDV [33,36,37]. However, our study focused only in sequences of the capsid protein VP60 and not in full-genome sequences. We further attempted to estimate for the first time the nucleotide substitution rate and the tMRCA of EBHSV, setting EBHSV emergence in the 1970s. Our results were in line with a recent estimation for RHDV, which proposes dates coincident with emergence only a few years before the first description in China in 1984 [32,60]. Inclusion of more EBHSV sequences from other European countries would certainly improve our estimation, since Eden and co-workers [60] showed that these estimations are sample-dependent and small changes in sample composition have an important impact on the final results.

Regarding the antigenic characterization of EBHSV strains, a scenario of arms race between host and pathogen can be hypothesized based on the results obtained. The replacement of Lineage A by Lineage B was likely accompanied by an increased fitness of the latter. Amino acid substitutions in previously recognized EBHSV epitopes occurred as a result of host immune response and consequent selection pressure from the host, leading to a response of immune evasion from the pathogen by epitope modification. This scenario is further supported by the existence of sites in the exposed regions of EBHSV capsid protein that are hypothetically under positive selection. Indeed, these sites might be involved in the arms race between host and pathogen. A similar finding was observed in the RHDV capsid protein [61,62].

The hypothetical positively selected sites identified in our study are located in exposed regions of the capsid, mostly in the P2 subdomain. For RHDV, variation in the amino acids of the P2 subdomain relates to HBGAs binding specificity [63]. Thus, the

variability in amino acids of the EBHSV capsid, likely under positive selection pressure, might influence receptor binding. An effective binding to host cells is crucial for virus entry and replication, and usually viruses achieve this by using cell surface molecules as attachment factors. Previous works disclosed that HBGAs A, B and H type 2 are attachment factors for RHDV [64,65], and fucosylated glycans were associated with RHDV infection [66]. Therefore, given the similarities between these two viruses, we attempted to characterize the glycans used by EBHSV to have a foothold on host cells.

EBHSV seems to bind to terminal *N*-acetylglucosamine (GlcNAc) residues and to heparin, which is a glycan related to heparan sulfate. The absence of a specific relation between ABH phenotype and EBHSV binding set EBHSV apart from RHDV in terms of host binding. Terminal GlcNAc residues were shown to be important for the attachment of other viruses [67,68], and heparan sulfate is used by many viruses, including members of the *Caliciviridae* family [e.g. 69,70-72]. Terminal GlcNAc residues are present in the duodenum of rabbits [64] whilst heparan sulfate distribution on tissues is not compatible with EBHSV binding patterns. Thus, the role of heparan sulfate in EBHSV attachment remains to be assessed. The absence of the B antigen in the upper respiratory and digestive tissues of hares (not fully confirmed due to the small number of hares available for this study) might help to explain why EBHSV evolved towards the usage of different attachment factors.

Why some *Lepus* species are susceptible to EBHSV while others are not is still uncertain. For example, the Iberian hare (*L. granatensis*) is not susceptible to EBHSV, despite (i) the high number of hares analyzed (C. Gortázar, personal communication), (ii) having a contact zone with the main host of EBHSV, the European brown hare (*L. europaeus*) [73], and (iii) European brown hares from Spain being susceptible to EBHSV [74]. Consistent with this, no antibodies against EBHSV were detected in Iberian hares (P. J. Esteves, personal communication). In our study, no differences in the binding of EBHSV to *L. granatensis* and *L. europaeus* tissues were observed that may account for the lack of EBHS cases in the Iberian hare. Therefore, factors other than the ability to attach to host cells must be involved, such as other steps of the virus life cycle or the limited contact between these species that does not allow the virus to infect Iberian hares.

The origins of lagoviruses

The high mutation rates of RNA viruses such as RHDV and EBHSV make them useful models to study evolution. One of the main aims of this thesis was to contribute to a better understanding of the evolutionary mechanisms of lagoviruses. Currently, two hypotheses for the emergence of pathogenicity in lagoviruses have been put forward: the evolution from a non-pathogenic virus or a species jump, each supported by several lines of evidence [75-83].

Our report of a cross-species infection of G1 in hares corresponds to the earliest evidence of a cross-species transmission in lagoviruses. Two strains presenting 6 % nucleotide divergence were able to infect hares, in different years and locations. Why RHDV was able to cross the species boundaries twice independently but was not able to adapt to the new host has not yet been disclosed. However, the strong selection pressure RHDV might be subject to can increase the likelihood of disease emergence in a novel host species. Our study, together with the recent evidences of EBHSV infection in *Sylvilagus* and of RHDV2 infection in *Lepus* species [84-86], showed that species jumps are possible in lagoviruses, and support the hypothesis of a species jump at the origin of pathogenicity in lagoviruses.

The binding of pathogenic and non-pathogenic viruses to all leporids, especially to *Sylvilagus*, also reinforces the hypothesis of a species jump from *Sylvilagus floridanus* after its introduction into Europe [87]. Although lagoviruses bind to the same tissues of the potential hosts (epithelial layer of trachea and Lieberkühn crypts in the duodenum), the affinity to different glycans varies. While non-pathogenic strains prefer the B type 2 antigen, as reported for the classical RHDV [64,65], RHDV2 has no preference among the HBGAs. These differences might not only be related to an adaptation to host evolution, but also to the ability of RHDV2 of infecting new hosts, such as hares. On the other side, our results may also support the evolution from non-pathogenic lagoviruses, since the non-pathogenic strains tested used the same attachment factors as RHDV.

2. Future perspectives

The research associated with this thesis shed light on several aspects of lagoviruses' evolution. However, as a consequence of this work, there are also many questions that were raised and remain unanswered. From that, future lines of work can be delineated that should be addressed in future research:

1. What was the role of recombination in the emergence of lagoviruses?

One of the main findings of this research was the existence of recombination in the new RHDV variant. Former studies indicate that recombination is present in the first pathogenic strains of RHDV [33], thus it is possible that recombination also played a role in the emergence of the new variant and in the expansion of host range. The analysis of RHDV2 strains from different regions and time points might help to disclose the events at the origin of this new variant.

2. Are there any differences in the fitness of the recombinant RHDV2 forms?

In the Iberian Peninsula, RHDV2 currently circulates (at least) as non-recombinant, as G1/RHDV2 recombinant and as non-pathogenic/RHDV2 recombinant. No data exist on the relative fitness of all these forms, a feature that will be interesting to assess. In order to do that, experimental infections would be needed to evaluate infection rates, clinical evolution of RHD and mortality rates for each form, as well as determination of virus replication efficiency through quantitative real-time PCR. Likewise, analysis of strains recovered from rabbits found dead in the field would allow to investigate the relationship of each RHDV2 form with the time of the year, the geographical location and the mortality.

3. *Are there non-pathogenic strains circulating in the Iberian Peninsula?*

The detection of recombining strains between non-pathogenic non-structural proteins and RHDV2 structural proteins in Portugal indicates that non-pathogenic strains must circulate in Iberian Peninsula, albeit never detected. The existence of non-pathogenic strains circulating in other regions of Europe [77,78] further reinforces the possibility of an Iberian non-pathogenic calicivirus. We propose to search for the non-pathogenic Iberian strains, by screening rabbit samples following the protocol described in Strive *et al.* [79]. The detection and further analysis of these non-pathogenic strains will uncover the genetic variability of lagoviruses and complete the information available, which will in turn help to disclose how they emerged as pathogenic forms.

4. *How are wild rabbit populations responding to the new RHDV variant?*

The devastating effects of the new RHDV variant in Iberian Peninsula and in Azores were discussed in this thesis. The response of wild rabbit populations is particularly important in the subspecies *O. c. algirus*, more geographically restricted. The resulting data of this thesis reinforce the importance of monitoring wild rabbit populations and evaluate the impact of RHDV2. At the moment, monitoring with systematic collection of samples constitutes the most effective measure to determine RHDV2 epidemiology and control rabbit populations. After the implementation of this system, conservation planning should be redefined accordingly.

5. *Will RHDV2 evolve similarly in Azores and in mainland Portugal?*

The co-evolution process results from the interactions between the virus and its host. Thus, the evolution of a virus is dependent on the nature of the populations in which it is circulating. The occurrence of similar strains of RHDV2 in mainland Portugal and in Azores at the same time is an exceptional opportunity to study how the virus will evolve in the two regions in response to different environmental and biotic factors. Similarly, possible alterations in the immune system of rabbits should be studied and compared.

6. How to continue improving knowledge for EBHSV?

Although this work brings new contributions to the knowledge of EBHSV evolution and interaction with its host, the results on EBHSV binding to host cells require further supporting evidence and clarification. The lack of a proper cell culture system to make EBHSV grow *in vitro* impairs faster advances in the area. Furthermore, detection and characterization of non-pathogenic forms of EBHSV would be important to complete the information on EBHSV, and to study potential past recombination events that led to the emergence of more virulent strains. In order to achieve that, full-genome sequences should also be obtained, instead of only partial fragments that might mask the existence of recombination.

7. What is the impact of EBHSV in the genetic variation of the host?

The secondary structure prediction of EBHSV capsid protein is now available. Therefore, regions and amino acids of the EBHSV capsid protein that are more likely to establish contact with the host cell surface are easily identified. On the host side, the cell surface molecules that participate in the attachment of EBHSV to the cells, as well as components of the immune system, may foresee host candidate genes to be under selective pressure. Integration of our data with evolutionary analysis of the host will help to disclose which host genes are involved in the arms race.

8. How can we predict future host shifts?

The nature and genetic details of host shifts are still unresolved: for example, it is unclear if the mutations found in the new host result from *de novo* mutations or standing variation in the original host [88]. Recombination might help host jumps, but it does not appear central to the cross-species transmission [39]. It would be interesting to study the nature of RHDV and EBHSV strains that were able to overcome the species boundaries, to determine the existence of recombination or other features that might distinguish them, such as specific sites in the sequence or positively selected codons. Likewise, the study of the factors that impair some lagoviruses to infect species other than their natural

host(s) may provide useful insights into how leporids are able to overcome the lagovirus infection.

9. How can we integrate research in lagoviruses with advances in scientific techniques?

Although in the last years we developed an efficient genome-walking amplification process of lagoviruses, we intend to start taking advantage of the high-throughput sequencing technologies that allow the generation of a very large volume of genetic data. This includes whole-genome sequences of several host species, such as the rabbit, but also of the viruses. Next Generation Sequencing technologies, when implemented, have the potential of being faster and cheaper than our current approach.

3. References

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