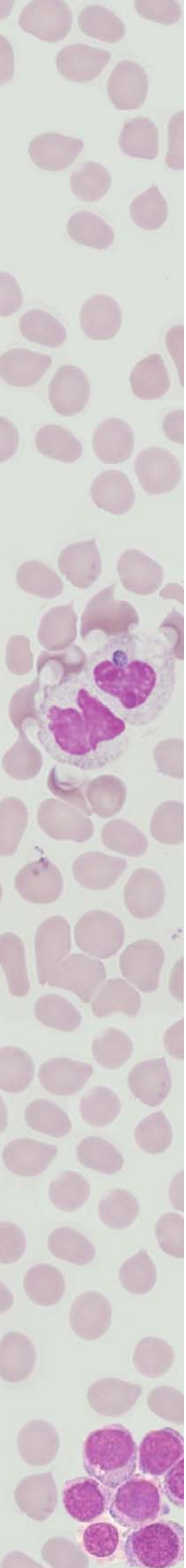


UNIVERSIDADE NOVA DE LISBOA

INSTITUTO DE HIGIENE E MEDICINA TROPICAL

# *Anaplasma phagocytophilum* and human granulocytic anaplasmosis in Portugal

Ana Sofia Pereira dos Santos

Dissertação apresentada ao Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa, para obtenção do Grau de Doutor no Ramo de Ciências Biomédicas, Especialidade de Microbiologia

LISBOA

2007

Neutrophil with *Anaplasma phagocytophilum* morulae. Pheripheral blood smear stained with Diff-Quik. Photograph kindly provided by Professor J Stephen Dumler, The Johns Hopkins University School of Medicine, Baltimore.

Neutrófilo com uma mórula de *Anaplasma phagocytophilum*. Esfregaço de sangue periférico corado com Diff-Quik. Fotografia gentilmente cedida pelo Professor J Stephen Dumler, The Johns Hopkins University School of Medicine, Baltimore.

Este trabalho foi financiado pela Fundação para a Ciência e a Tecnologia através da atribuição da bolsa de doutoramento refª SFRH/BD/8610/2002.

## ORIENTAÇÃO:

Doutora Fátima Bacellar
Investigadora Principal Aposentada
Centro de Estudos de Vectores e Doenças Infecciosas
Instituto Nacional de Saúde Dr. Ricardo Jorge, Lisboa

Professor John Stephen Dumler
Professor Associado
Division of Medical Microbiology, Department of Pathology
The Johns Hopkins University School of Medicine, Baltimore

## COMISSÃO TUTORIAL:

Doutora Margarida Collares Pereira
Investigadora Principal com Habilitação
Instituto de Higiene e Medicina Tropical
Universidade Nova de Lisboa, Lisboa

Professor Doutor Paulo Almeida
Professor Associado
Instituto de Higiene e Medicina Tropical
Universidade Nova de Lisboa, Lisboa

Doutora Fátima Bacellar
Investigadora Principal Aposentada
Centro de Estudos de Vectores e Doenças Infecciosas
Instituto Nacional de Saúde Dr. Ricardo Jorge, Lisboa

Na presente dissertação incluem-se resultados que foram ou estão a ser alvo de publicação em co-autoria. Para efeitos do disposto no nº1 do Despacho nº2303/2000 do Regulamento de Programas de Doutoramento do Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa (Diário da República, 2ª série, nº23, de 28 de Janeiro de 2000), o autor da dissertação declara que interveio na concepção e execução do trabalho experimental, na interpretação dos resultados e na redacção dos manuscritos publicados, no prelo ou que aguardam submissão.

Lisboa, 11 de Setembro de 2007

Ana Sofia Pereira dos Santos

*a Arminda*

# ACKNOWLEGEMENTS / AGRADECIMENTOS

Ao Instituto Nacional de Saúde Dr. Ricardo Jorge (INSA) a possibilidade de realizar os trabalhos que permitiram a elaboração da presente dissertação;

À Fundação para a Ciência e a Tecnologia, a atribuição da bolsa de doutoramento (refª SFRH/BD/8610/2002), sem a qual teria sido impossível a realização deste projecto;

Ao Professor Doutor Armindo Filipe e Doutora Maria Sofia Núncio, responsáveis pelo Centro de Estudos de Vectores e Doenças Infecciosas, Instituto Nacional de Saúde Dr. Ricardo Jorge (CEVDI/INSA), em diferentes fases do meu percurso, pelo apoio e incentivo que tenho merecido desde os primeiros passos no mundo da Ciência e pela disponibilização de todos os recursos do Centro para o bom andamento da parte experimental desta dissertação;

À Doutora Fátima Bacellar, minha orientadora no CEVDI/INSA e mentora, a quem devo a minha formação, o apoio incondicional e a amizade que tem sido uma constante desde que as nossas vidas se cruzaram;

To Professor John Stephen Dumler, my sponsor at The Johns Hoopkins University School of Medicine, for all the knowledge, support and stimulation. I am grateful for knowing you and honoured to be able to share some of your expertise in *A. phagocytophilum* field.

À Doutora Margarida Collares Pereira e ao Professor Doutor Paulo Almeida, por terem aceite ser o elo de ligação ao Instituto de Higiene e Medicina Tropical (IHMT), e que com as suas sugestões, apoio e incentivo simplificaram várias etapas deste trabalho;

Ao Professor Doutor Virgílio do Rosário e Professora Doutora Maria Amélia Grácio, anterior e actual Presidente do Conselho Científico do IHMT, pelo acolhimento institucional e disponibilidade na resolução de todas as questões académicas determinantes para a boa consecução deste projecto;

Ao Mestre João Lavinha, ex-Director do INSA e presentemente responsável pelo Centro de Genética Humana do mesmo Instituto, por acreditar e apoiar todos os projectos que me proponho desenvolver;

To all the Researchers that since a long time have collaborated with CEVDI/INSA in Anaplasmatacea field, from which the work developed in this dissertation has beneficiated, especially to Jackie Dawson from CDC - Atlanta, Professor Didier Raoult and Philipe Brouqui from Unité des Rickettsie - Marseille, José António Oteo and Arantza Portillo from Hospitales San Millán-San Pedro de La Rioja – Logroño.

Também aos Investigadores portugueses, Professor Doutor João David de Morais, Doutora Susana da Franca, Professor Doutor Victor Caeiro e Professor Doutor António José do Santos Grácio, com quem tive o privilégio de colaborar em várias etapas do meu percurso científico.

A presente dissertação é ainda testemunho da cooperação, dedicação e amizade de muitos a quem gostaria de expressar o meu profundo reconhecimento, como os colegas e amigos do CEVDI/INSA, do Dumler's lab, do Laboratório de Microbiologia e Ecologia e da Unidade de leptospirose e borreliose de Lyme;

Por último, aos primeiros em matéria de partilha de sofrimento e alegrias nestas etapas da minha vida, aos meus pais, em nome de toda a família, amigos e ao Jorge.

Foi por vós que aqui cheguei…

# ABSTRACT

*Anaplasma phagocytophilum* and granulocytic anaplasmosis are of increasing interest to the scientific community as indicated by the expanding number of reports published in the past two decades, and especially since the emergence of the first cases of human granulocytic anaplasmosis (HGA). The growing recognition of the Public Health importance of *A. phagocytophilum* in North America and in Europe, along with its recent detection in Portugal has signalled the need for more detailed studies that address the emergence of HGA and its causative agent in our country.

Initially based on an methodological training in research units dedicated to Anaplasmataceae, this work enabled the transfer of technology as currently applied to *A. phagocytophilum* research and made possible the development of a new line of investigation at *Centro de Estudos de Vectores e Doenças Infecciosas*, *Instituto Nacional de Saúde Dr. Ricardo Jorge* (*CEVDI/INSA*). By establishing the foundations for concerted study on *A. phagocytophilum* and HGA, the work described herein has facilitated development of a broad approach toward fundamental issues in Anaplasmataceae ecobiology and disease by focusing attention on identification of potential ixodid vectors, mammals likely to be involved in the infectious agent's life cycles either as reservoirs or affected hosts, and garnered the evidence indicating the potential for human exposure in Portugal.

The studies conducted in ixodid ticks proves the involvement of two *Ixodes* species in *A. phagocytophilum* cycles, including *Ixodes ricinus* on Madeira Island and *I. ventalloi* on the mainland. The detection of *A. phagocytophilum* DNA in *I. ricinus* reinforces prior studies and suggests its persistence on Madeira Island. This thesis also adds new data to the understanding of the natural history of *A. phagocytophilum* by providing the first evidence of infections in *I. ventalloi* ticks. The fact that some infected arthropods infest domestic cats not only mandates the inclusion of these mammals on the national list of vertebrate hosts parasitized by *I. ventalloi* ticks, but also shows their potential complicity in *A. phagocytophilum* maintenance. Moreover, molecular data shows the existence of *A. phagocytophilum* variant genotypes in Portuguese ticks. Partial gene sequences from infected ticks demonstrates nucleotide polymorphisms that support a close relationship of *A. phagocytophilum* on Madeira Island *I. ricinus* to North American strains isolated from humans as well as genotypes detected in Central and Northern Europe. Yet, these variants diverge from those found in mainland *I. ventalloi*, which represents a new genotype of undetermined pathogenicity.

Serological evidence of exposure to *A. phagocytophilum* or a close related agent is shown in *Mus spretus* mice, horses, and dogs in mainland. Molecular analysis of biological samples from these animal populations provides the first definitive evidence of *A. phagocytophilum* active infection in Portuguese vertebrates with detection of its DNA in one seropositive horse from mainland Portugal, where the *A.*

*phagocytophilum* genotype found is closely related to strains isolated from humans, suggesting the potential for HGA in Portugal. This thesis further extends study to identification of another closely related *Anaplasma* species, and its potential for serological cross-reactions with *A. phagocytophilum*, as evident with the detection of *A. platys* DNA in seropositive dogs. These data also underscore the importance of veterinarians maintaining vigilance for detection of not only granulocytic anaplasmosis but also canine infectious cyclic thrombocytopenia as causes of tick-borne diseases in Portugal.

Both prospective and retrospective serological and molecular investigations of human exposure to *A. phagocytophilum* were performed on samples received at *CEVDI/INSA* for the laboratory diagnosis of patients with suspected tick-borne diseases during 2000-2006. The results provide evidence for seropositive Portuguese patients, including cases that fulfil serological criteria for HGA, although active infections were not detected. Moreover some seropositive patients had additional evidence of other tick-borne agents or related bacteria infections, including Lyme borreliosis, Q fever and bartonellosis. Although possibly false positive cross-reactions to shared antigens, these reactions potentially could be the result of active dual infections, or past exposure to several agents transmitted by *Ixodes* species. Overall, these results argue for continued development of improved *A. phagocytophilum* diagnostics, especially direct detection techniques, and integrated analysis of diagnostic tests for patients with suspected *Ixodes*-borne disease.

Although many aspects introduced and explored here will require expanded and more detailed investigations, this thesis contributes positively to a fundamental understanding of the extent to which *A. phagocytophilum* occurs in Portugal and its potential as a disease agent. It is hoped that these beginning studies will help to delineate new lines of research that more fully address granulocytic anaplasmosis and other emerging *Ixodes*-borne diseases.

# RESUMO

*Anaplasma phagocytophilum* e anaplasmose granulocítica têm vindo a merecer um crescente interesse na comunidade científica, facto demonstrado pelo crescente número de publicações que surgiram nas duas últimas décadas, particularmente desde que foram documentados os primeiros casos de anaplasmose granulocítica humana (AGH). O reconhecimento da importância de *A. phagocytophilum* em termos de Saúde Pública na América do Norte e na Europa, associado à recente detecção do agente em Portugal, pôs em evidência a necessidade de estudos mais detalhados sobre a emergência deste Anaplasmataceae e de AGH no nosso país.

Inicialmente baseado num treino metodológico em Unidades de Investigação especializadas em Anaplasmataceae, o presente trabalho permitiu a transferência da tecnologia correntemente aplicada ao estudo de *A. phagocytophilum* e consequentemente o desenvolvimento desta linha de investigação no Centro de Estudos de Vectores e Doenças Infecciosas, Instituto Nacional de Saúde Dr. Ricardo Jorge (CEVDI/INSA). Ao estabelecer as bases para um estudo concertado em *A. phagocytophilum* e AGH, este trabalho tornou possível uma abordagem abrangente a aspectos essenciais da ecobiologia deste Anaplasmaceae e da doença que lhe está associada, focando-se na identificação de potenciais ixodídeos vectores, de mamíferos envolvidos no ciclo natural do agente, quer como reservatórios quer como hospedeiros susceptíveis, recolhendo evidências que apontam para a possibilidade de exposição do homem a este agente em Portugal.

Os estudos levados a cabo nos ixodídeos demonstram o envolvimento de duas espécies de *Ixodes* no ciclo de *A. phagocytophilum*, concretamente *Ixodes ricinus* na Ilha da Madeira e *I. ventalloi* no continente. A detecção de ADN do agente em *I. ricinus* vem reforçar estudos anteriores, sugerindo a sua persistência na Ilha da Madeira. Esta tese acrescenta ainda um novo dado ao conhecimento da história natural de *A. phagocytophilum*, com a primeira detecção de infecção em *I. ventalloi*. O facto de alguns dos artrópodes infectados terem sido obtidos a partir de gatos domésticos, não só acrescenta este mamífero à lista nacional de hospedeiros vertebrados parasitados por *I. ventalloi* mas sugere também o seu potencial envolvimento no ciclo de transmissão de *A. phagocytophilum*. A análise molecular dos dados obtidos evidencia ainda a existência de diferentes genótipos deste Anaplasmaceae nos ixodideos portugueses. As sequências nucleotídicas obtidas sugerem a proximidade genética entre *A. phagocytophilum* detectado em *I. ricinus* na Ilha da Madeira e estirpes isoladas de humanos na América do Norte, bem como de sequências detectadas na Europa Central e do Norte. No entanto estes genótipos divergem das sequências nucleotídicas obtidas a partir do *I. ventalloi* no continente, que representa um novo genótipo de patogenia não determinada.

São também apresentadas evidências serológicas da exposição a *A. phagocytophilum* ou a outro agente antigenicamente semelhante em ratinhos *Mus spretus*, cavalos e canídeos domésticos no continente. A análise molecular das amostras biológicas obtidas a partir das populações em estudo permitiu a detecção da primeira infecção activa por *A. phagocytophilum* em vertebrados portugueses, com a identificação de ADN do agente num dos cavalos seropositivos. Sendo as sequências nucleotídicas obtidas semelhantes à das estirpes associadas aos casos de infecção em humanos, é reforçada uma vez mais a possibilidade de AGH ocorrer em Portugal. Esta tese refere ainda a identificação de outra Anaplasmataceae filogenicamente próxima de *A. phagocytophilum* - *A. platys*, detectada em canídeos seropositivos, evidenciando a possibilidade de ocorrerem reacções cruzadas entre estes dois agentes. Estes dados salientam a importância da vigilância em termos veterinários da ocorrência não só de anaplasmose granulocítica mas também de trombocitopénia cíclica infecciosa canina como causa de doença associada a ixodídeos em Portugal.

Para averiguar a possibilidade de exposição humana a *A. phagocytophilum* foram realizados estudos serológicos e moleculares retrospectivos e prospectivos, em amostras biológicas recebidas no CEVDI/INSA durante 2000-2006 para o diagnóstico laboratorial de pacientes com suspeita de doença associada a ixodídeos. Os resultados provam a existência de reacções serológicas positivas na população estudada, incluindo alguns casos que satisfazem os critérios serológicos para definição de AGH, embora sem evidência de infecção activa. Adicionalmente, alguns dos doentes seropositivos apresentaram evidência de infecção causada por outros agentes transmitidos por ixodídeos ou por bactérias relacionadas, nomeadamente borreliose de Lyme, febre Q e bartonelose. Embora estes resultados possam ser interpretados como falsos positivos resultantes de reacções cruzadas, a possibilidade de se tratarem de co-infecções ou exposições anteriores a vários agentes transmitidos por ixodídeos do género *Ixodes* também deverá ser considerada. Em suma, estes resultados apontam para a necessidade de desenvolver o diagnóstico de *A. phagocytophilum*, nomeadamente no que diz respeito à aplicação de técnicas directas de detecção do agente, bem como à integração dos dados do diagnóstico laboratorial em casos de suspeita de doença associada a *Ixodes* spp.

Embora muitos dos aspectos focados neste trabalho requeiram uma investigação mais detalhada, esta tese contribui positivamente para a compreensão das questões fundamentais relacionadas com a ocorrência de *A. phagocytophilum* e o seu potencial como agente de doença em Portugal. Termina-se com a esperança de que estes primeiros passos ajudem a delinear novas linhas de investigação no âmbito do estudo da anaplasmose granulocítica e outras doenças emergentes associadas a espécies do género *Ixodes*.

# GENERAL INDEX

# FIGURES INDEX

# TABLES INDEX

# LIST OF ABBREVIATURES AND ABBREVIATIONS

| | |
|---|---|
| *ankA* | Gene encoding AnkA |
| AnkA | The 153-160 kDa protein with several tandemly repeated ankyrin motifs |
| ATCC | American Type Culture Collection |
| AT-rich | Adenine-timine rich |
| BF | Boutonneuse fever [also referred as Mediterranean spotted fever (MSF)] |
| bp | Base pair |
| CDC | Centers for Disease Control and Prevention |
| *CEVDI* | *Centro de Estudos de Vectores e Doenças Infecciosas* |
| CICT | Canine Infectious cyclic thrombocytopenia |
| CSF | Cerebrospinal fluid |
| DH82 | Canine macrophage-like cell line |
| DNA | Desoxiribonucleic acid |
| EDTA | Ethylenediaminetetraacetic acid |
| EGA | Equine granulocytic anaplasmosis |
| ELISA | Enzyme-linked Immunosorbent assay |
| FBS | Fetal bovine serum |
| FICT | Fluorescein isothocyanate |
| *flgB* | Flagellin gene |
| GA | Granulocytic anaplasmosis |
| G+C | Guanine + cytosine |
| *gltA* | Citrate synthase gene |
| *groESL* | Heat shock operon |
| GroESL | Heat shock proteins, including 10-20 kDa GroES and 58-65 kDa GroEL (also named GroL) that act as chaperonins |
| HB | Human babesiosis |
| HGA | Human granulocytic anaplasmosis |
| HGE | Human granulocytic ehrlichiosis (presently human granulocytic anaplasmosis) |
| HL-60 | Human promyelocytic leukaemia cell line |
| rHL-60 | HL-60 cells induced to differentiate into functional granulocytes |
| HME | Human monocytic ehrlichiosis |
| *ICNB* | *Instituto de Conservação da Natureza e da Biodiversidade* |

| | |
|---|---|
| IFA | Immunofluorescence assay |
| IgG, IgM | Immunoglobulin G, immunoglobulin M |
| *INSA* | *Instituto Nacional de Saúde Dr. Ricardo Jorge* |
| kDa | Kilodalton |
| LB | Lyme borreliosis |
| LPS | Lipopolysaccharide |
| MEM | Minimum essential medium |
| MSF | Mediterranean spotted fever [also referred as boutonneuse fever (BF)] |
| *msp2* | Surface proteins gene family (also *p44*) |
| MSP2 | Surface proteins family (also P44) |
| NETSS | National Electronic Telecommunications Systems for Surveillance |
| *OmpA* | Outer membrane protein A gene |
| *OmpB* | Outer membrane protein B gene |
| *p44* | Surface proteins gene family (also *msp2*) |
| P44 | Surface proteins family (also MSP2) |
| PBS | Phosphate buffered saline |
| PCR | Polymerase chain reaction |
| RMSF | Rocky Mountain spotted fever |
| *rrs* | 16S rRNA gene |
| Salp | Ticks salivary gland protein |
| *s.l.* | *Sensu lato* |
| *s.s.* | *Sensu stricto* |
| TBE | Tick-borne encephalitis |
| TBF | Tick-borne fever (also referred as pasture fever) |
| *TNM* | *Tapada Nacional de Mafra* |
| US | The United States of America (also abbreviated as USA) |
| vero E6 | Epithelial-like cell line obtained from kidney epithelial cells of the african green monkey (*Cercopithecus aethiops*) |
| WB | Western blot |

# INTRODUCTION

# OUTLINE AND OBJECTIVES

Ticks are obligate hematophagous acarines that present widespread distribution and parasitize a broad range of terrestrial vertebrates, including amphibians, reptiles, birds, domestic and wild mammals and humans. Its implication in infectious disease was first evident in veterinary medicine when *Boophilus annulatus* was linked to Texas cattle fever via transmission of *Babesia bigemina* (Smith & Kilbourne, 1893). In the beginning of the 20th century, ticks were additionally implicated in human disease with the description of tick-borne relapsing fever, caused by *Borrelia duttonii*, and its association with *Ornithodoros moubata* bites (Dutton & Todd, 1905), as well as recognition of the role that *Dermacentor andersoni* plays as vectors of *Rickettsia rickettsii*, the agent of Rocky Mountain spotted fever (Ricketts, 1909). Since then, ticks were linked to several other protozoal, bacterial and viral infections. The last three decades in particular have revealed the emergence of an increasing number of human tick-borne diseases, mostly due to the expanding knowledge of ticks, their pathogenic potential and the development of more reliable diagnostic tools.

Human infections caused by bacteria belonging to the family Anaplasmataceae represent some of the best examples of newly emergent tick-borne diseases. Some of these agents have been long known as veterinary pathogens but their implication in human cases has only recently been recognized, such as for *Anaplasma phagocytophilum* the etiologic agent of human granulocytic anaplasmosis (HGA). Disease caused by this microorganism was first recognized in 1932 in European sheep and cattle (Gordon *et al*., 1932), and a similar agent was also later found to be the cause of equine pathology in the United States of America (US) (Gribble, 1969; Stannard *et al.*, 1969). However, only in the 1990's were the first cases of human infection described in US (Bakken *et al*., 1994; Chen *et al*., 1994) and in Europe (Petrovec et al, 1997). At present, HGA stands out as the most prevalent human disease caused by an Anaplasmataceae member, due to the number of cases that are reported annually in US; in some areas of the country HGA is the second most frequent vector-borne disease following Lyme borreliosis (LB)[1]. Moreover, it is suggest that *A. phagocytophilum* is found broadly across several countries in Europe, Middle East and Asia, raising the possibility of human and animal infections over the same geographic regions.

[1] Lyme borreliosis (LB) is a tick-borne disease caused by the eubacteria *Borrelia burgdorferi senso lato (s.l.)*.

Although the number of HGA cases outside the US is still limited, the possibility that this zoonosis is under-diagnosed owing to a lack of physician awareness or inavailability of current laboratory diagnostics is controversial and of great concern.

As HGA has now been readily recognized in other European countries, this thesis was undertaken to contribute to the fuller understanding of *A. phagocytophilum* ecobiology, with an emphasis on its potential importance to human health in Portugal.

In summary, the general aims of this thesis are:

- To investigate the role of Portuguese tick species as vectors of *A. phagocytophilum*;
- To search for potential reservoirs and affected vertebrate hosts in Portugal;
- To ascertain the importance of *A. phagocytophilum* in human disease;
- To develop methodological and technical approaches applied to HGA laboratory diagnosis;
- To isolate and/or characterize *A. phagocytophilum* strains circulating in the country.

## DISSERTATION PLAN

The thesis is organized in six chapters:

The first chapter is the introduction to the theme providing general information about tick-borne bacteria belonging to the family Anaplasmataceae and a broad updated perspective of *A. phagocytophilum* and its role in HGA.

Chapter two reflects the collaborative work focused on Anaplasmataceae field, developed during the technology exchange and the acquisition of tools and knowledge that provided the basis for developing the proposed research plan.

Chapter three is devoted to the study of *A. phagocytophilum* vectors. It describes molecular-based analyses of Anaplasmataceae within several tick species obtained either questing or parasitizing vertebrates in several areas of the country.

Chapter four explores the possibility of non-human vertebrate exposure to *A. phagocytophilum* supported by serological and molecular-based analyses of biological samples obtained from both wild and domestic animals.

Chapter five addresses the epidemiological basis of HGA in Portugal based on serologic studies of patients with suspected or confirmed tick-borne diseases and in the application of serologic testing in routine diagnosis performed at the *Centro de Estudos de Vectores e Doenças Infecciosas*, *Instituto Nacional de Saúde Dr. Ricardo Jorge* (*CEVDI/INSA*).

Finally, chapter six summarises the results presented and discussed in previous chapters in an integrated analysis, highlighting their implications in Public Health and delineating future research objectives.

# CHAPTER I

## STATE OF THE ART

# 1.1. FAMILY ANAPLASMATACEAE

## 1.1.1. DEFINITION

Members of the family Anaplasmataceae are non-motile, obligatory intracellular Gram-negative bacteria that reside in a membrane-bound cytoplasmic vacuole of the host cell, either singly or more often in characteristic microcolonies resembling mulberries, termed morulae (Latin *morum* = mulberry, Figure 1). They are represented by fifteen or sixteen species, classified in five distinct genera: *Aegyptianella*, *Anaplasma*, *Ehrlichia*, *Neorickettsia* and *Wolbachia* (Table 1). (Garrity *et al.*, 2004; Dumler *et al.*, 2005b).

Most Anaplasmataceae are etiologic agents of worldwide veterinary diseases, some of which are now also regarded as emerging human pathogens. The only exception is the genus *Wolbachia* that includes arthropod and nematode endosymbionts, but that may be involved in the pathogenesis of filiariasis (Hoerauf *et al.*, 2001; Taylor *et al.*, 2005). In general, the biological cycle of Anaplasmataceae involves the infection of invertebrates (*Wolbachia*), and incidentally also vertebrates (*Neorickettsia*) or both invertebrate and vertebrate hosts (*Aegyptianella*, *Anaplasma* and *Ehrlichia*). Unique cell tropisms are displayed by these agents and depending on bacterial species, endothelial and/or hematopoietic lineage cells (erythrocytes, monocytes/ macrophages, granulocytes or platelets) are specifically infected in vertebrate hosts. The most important biological, ecological and epidemiological features of Anaplasmataceae with medical importance are showed in table 2.

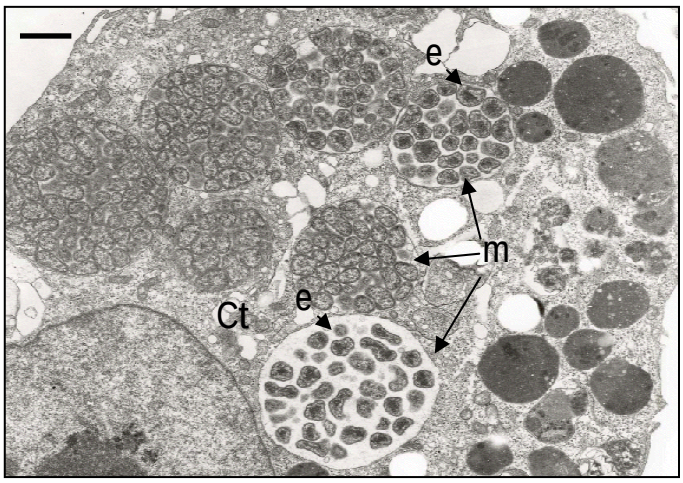



FIGURE 1. Electron photomicrograph of a canine macrophage-like cell (DH82, ATCC CRL-10389) infected with *Ehrlichia canis* (adapted from Popov *et al.*, 1998).

The cytoplasm (Ct) contains seven morulae (m) filled with ehrlichiae (e), showing coccoid to ellipsoidal, often pleomorphic shape. Bar 1µm.

TABLE 1. Current classification of the family Anaplasmataceae (adapted from Garrity *et al.*, 2004).

| Family Anaplasmataceae | | | | |
|---|---|---|---|---|
| Genus | | | | |
| *Anaplasma* | *Aegyptianella (incertae sedis)* | *Ehrlichia* | *Neorickettsia* | *Wolbachia* |
| *A. bovis*<br>*A. caudatum*<br>*A. centrale*<br>*A. marginale*<br>*A. platys*<br>*A. phagocytophilum* | *A. pullorum* | *E. canis*<br>*E. chaffeensis*<br>*E. ewingii*<br>*E. muris*<br>*E. ruminantium* | *N. helminthoeca*<br>*N. risticii*<br>*N. sennetsu* | *W. pipientis* |

## 1.1.2. PHYLOGENY AND TAXONOMY

The family Anaplasmataceae is taxonomically classified in the Domain Bacteria, Phylum Proteobacteria, Class "Alphaproteobacteria" and Order Rickettsiales. For many years, Anaplasmataceae were categorized based solely on morphological, ecological, epidemiological and clinical characteristics such as infected host cells, infected mammalian species, geographic location, and antigenic cross-reactivity (Philip, 1957; Moulder, 1974; Weiss & Moulder, 1984). The development of molecular tools for phylogenetic studies and the improvement of methods for the cultivation of obligate intracellular bacteria has helped clarify the exact phylogenetic positions of Anaplasmataceae, which in turn has lead to profound reorganization of this Family and other closely related *taxa* (Garrity *et al.*, 2004) (Figure 2).

The family Anaplasmataceae was first proposed in the seventh edition of Bergey's Manual of Determinative Bacteriology to include the genus *Anaplasma* composed of three species, *A. centrale*, *A. marginale* and *A. ovis* (Philip, 1957). Four additional genera, *Aegyptianella* (*A. pullorum*), *Eperythrozoon* (*E. coccoides*, *E. ovis*, *E. parvum, E. suis* and *E. wenyonii*), *Haemobartonella* (*H. canis*, *H. felis* and *H. muris*) and *Paranaplasma* (*P. caudatum* and *P. discoides*) were included in the revision that followed (Moulder, 1974). In the first edition of Bergey's Manual of Systematic Bacteriology *Paranaplasma* was assigned to the genus *Anaplasma*, and *P. caudatum* was renamed as *A. caudatum*, which represented the last categorization of this family based exclusively in phenotypic similarity (Weiss & Moulder, 1984).

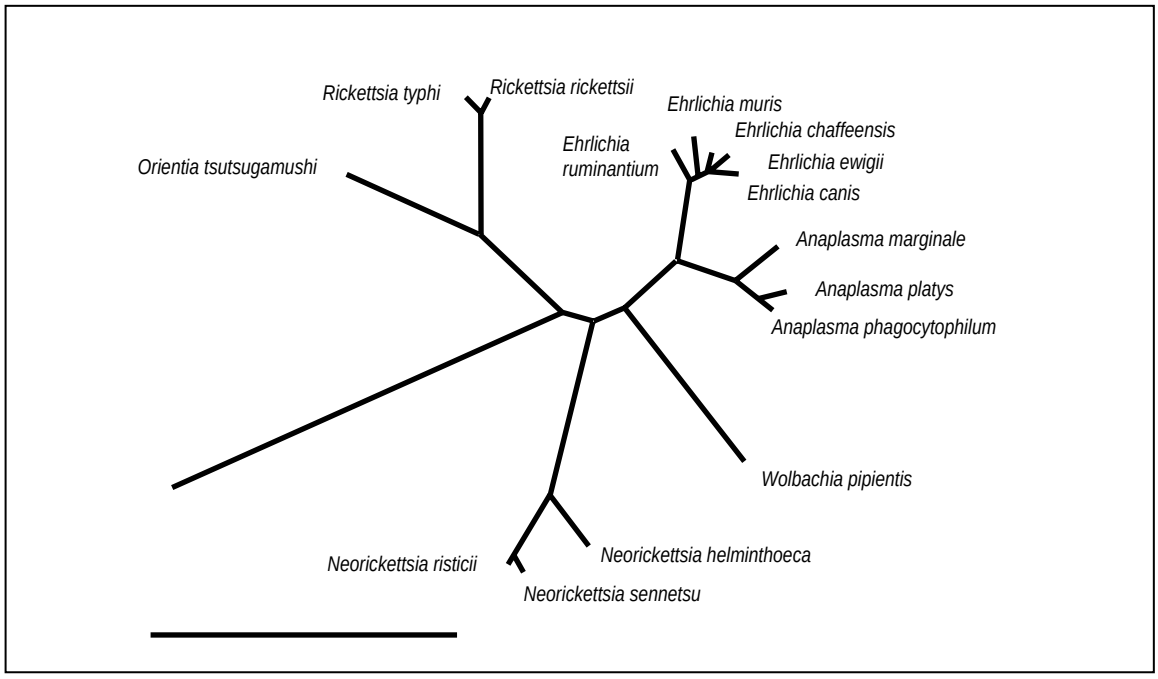
In the following years the analysis of conserved genes such as 16S rRNA (*rrs*), heat shock operon (*groESL*), and citrate synthase (*gltA*) sequences started to outline phylogenetic relationships of Anaplasmataceae and other closely related bacteria (Weisburg *et al.*, 1989, 1991; Dame *et al.*, 1992; O'Neill *et al.*, 1992; Wen *et al.*, 1995, 1996; Pretzman *et al.*, 1995; Rikihisa *et al.*, 1997; Zhang *et al.*, 1997; Sumner *et al.*, 2000; Inokuma *et al.*, 2001a, 2001b; Yu *et al.*, 2001). This approach has been widely used to both identify newly discovered bacteria as well as to redefine existing taxonomy, thus showing that the taxonomic classification of Anaplasmataceae needed to be restructured.

Molecular analysis has shown that *Eperythrozoon* and *Haemobartonella* species did not cluster with the other Anaplasmataceae, so their removal from the order Rickettsiales to Mollicutes was proposed (Rikihisa *et al.*, 1997). It has also been shown that some agents classified in the family Rickettsiaceae, especially those of the tribe *Ehrlichieae* and *Wolbachieae*, were closely related to *Anaplasma* (O'Neill *et al.*, 1992; Drancourt & Raoult, 1994; Wen *et al.*, 1995; Walker & Dumler, 1996; Sumner *et al.*, 2000; Inokuma *et al.*, 2001a, 2001b; Yu *et al.*, 2001). Based on an exhaustive analysis of *rrs*, *groESL* and surface protein gene sequences deposited in GenBank, Dumler and coworkers (2001) proposed a reorganization of the Order Rickettsiales. Concerning Anaplasmataceae, it was proposed: **i**) to include the closely related species of the family Rickettsiaceae (*Ehrlichia*, *Neorickettsia*, *Cowdria*, *Wolbachia*); **ii**) to expand the genus *Anaplasma* and incorporate *Ehrlichia bovis*, *Ehrlichia platys* and *Anaplasma phagocytophilum* (including *Ehrlichia phagocytophila*, *Ehrlichia equi* and human granulocytic ehrlichiosis [HGE] agent). The unification of *E. phagocytophila*, *E. equi* and HGE agent as a single species confirmed previous studies that suggested a close molecular (Chen *et al.*, 1994; Pretzman *et al.*, 1995; Goodman *et al.*, 1996; Sumner *et al.*, 1997; Zhang *et al.*, 1997; Inokuma *et al.*, 2001a, 2001b) and antigenic (Barlough *et al.*, 1995; Dumler *et al.*, 1995; Madigan *et al.*, 1995) similarity between those agents; **iii**) to fuse *Cowdria* with the genus *Ehrlichia* and place the sole species, *Cowdria ruminantium*, with the existing species *Ehrlichia canis*, *Ehrlichia chaffensis*, *Ehrlichia ewingii* and *Ehrlichia muris*; **iv**) to expand the genus *Neorickettsia* to include *Ehrlichia risticii* and *Ehrlichia sennetsu*; **v**) to exclude *Wolbachia persica* and *W. melophagi* from the genus *Wolbachia* and to include the genus *Wolbachia* with its sole member *Wolbachia pipientis*; **vi**) to retain provisional *Aegyptianella* as *Genus incertae sedis* due to the lack of molecular information.

Almost all the proposed reorganizations in family Anaplasmataceae were introduced in the second edition of Bergey's Manual of Systematic Bacteriology (Garrity *et al.*, 2001, 2002a, 2002b,

2003, 2004; Dumler *et al.*, 2005b). The heterogeneity of *W. pipientis* might also justify the revision of species concept with creation of additional *taxa* to include the distinct host-associated clades that have been described (Lo *et al.*, 2002; Fen *et al.*, 2006). Moreover, the *incertae sedis* classification of *Aegyptianella* has been called to question owing to its phenotypic similarities with *Anaplasma* spp., and later reinforced by genetic data (Rikihisa *et al.*, 2003). In future revisions new agents might be included in Anaplasmataceae, such as Candidatus *Neoehrlichia mikurensis* (*Ehrlichia walkerii*), *E. shimanensis*, and *Xenohaliotis californiensis* (Friedman *et al.*, 2000 ;Brouqui *et al.*, 2003; Kawahara *et al.*, 2004, 2006; Rikihisa, 2006a).

FIGURE 2. Current phylogeny and taxonomic classification of genera in the family Anaplasmataceae. The distance bar represents substitutions per 1,000 bp (adapted from Dumler *et al.*, 2005a).




## 1.1.3. HISTORICAL PERSPECTIVE OF HUMAN INFECTION

Our current knowledge of Anaplasmataceae started at the beginning of the 20th century with the descriptions of Theiler (1910) implicating *Anaplasma marginale* as the etiologic agent of anaplasmosis, a tick-borne disease of ruminants that is still associated to enormous worldwide annual economic losses. In the following years several other agents causing disease in domestic and wild animals were identified, such as *Anaplasma centrale* (Theiler, 1911); *Cowdria* (now

*Ehrlichia*) *ruminantium* (Cowdry, 1925), *Ehrlichia* (now *Anaplasma*) *ovis* (Lestoquard, 1924), *Ehrlichia phagocytophila* (now *Anaplasma phagocytophilum*) (Gordon *et al.*, 1932; Foggie 1951), *Ehrlichia canis* (Donatien & Lestoquard, 1935), *Ehrlichia* (now *Anaplasma*) *bovis* (Donatien & Lestoquard, 1936), *Neorickettsia helminthoeca* (Philip *et al.*, 1953), *Ehrlichia equi* (now *Anaplasma phagocytophilum*) (Gribble, 1969; Stannard *et al.*, 1969), *Ehrlichia* (now *Anaplasma*) *platys* (Harvey *et al.*, 1978), *Ehrlichia* (now *Neorickettsia) risticii* (Holland *et al.*, 1985; Rikihisa & Perry, 1985), *Ehrlichia ewingii* (Ewing *et al.*, 1971; Anderson *et al.*, 1992), *Ehrlichia muris* (Wen *et al.*, 1995) (Table 2).

In spite of abundant reports in the veterinary field, no case of human infection by Anaplasmataceae was described before the 1950s. Sennetsu fever, documented in Japan in 1954 (Misao & Kobayashi, 1955), was formally the first known human disease caused by an Anaplasmataceae member, although it now commands little medical significance. In fact, the mononucleosis-like illness caused by *Neorickettsia* (formerly *Ehrlichia*) *sennetsu* occurs only in limited areas of the Far East, rarely outside Japan and Malaysia and is usually mild, with no deaths having ever been reported. However, in the last decade of the 20th century, the scientific community's view of Anaplasmataceae infections changed dramatically with the description of two new severe to fatal forms of disease in United States (US). In 1986, human monocytic ehrlichiosis (HME) was reported from Central Arkansas (Maeda *et al.*, 1987). This disease was caused by a new species named *Ehrlichia chaffeensis*, closely related to the veterinary pathogens *E. canis* and *E. ewingii* (Anderson *et al.*, 1991; Dawson *et al.*, 1991) and later proven to be also infective for animals, as summarized in Paddock and Childs (2003). In the early 1990's, cases of human granulocytic anaplasmosis (HGA) (formerly human granulocytic ehrlichiosis [HGE]) were reported from Minnesota and Wisconsin (Bakken *et al.*, 1994; Chen *et al.*, 1994). The exact nature of the causative agent, originally named "HGE agent", remained uncertain for several years until its unification with the animal pathogens *E. phagocytophila* and *E. equi* into a single species designation, *Anaplasma phagocytophilum* (Dumler *et al.*, 2001; Garrity *et al.*, 2003). Additionally, two other Anaplasmataceae of well known veterinary importance were also described as a cause of human infection. In 1996, *E. canis* - the etiologic agent of canine monocytic ehrlichiosis was isolated from an apparently healthy man (Perez *et al.*, 1996; Unver *et al.*, 2001b), and later associated with several symptomatic cases (Perez *et al.*, 2006) in Venezuela. More recently in 1999 *E. ewingii*, the etiologic agent of canine granulocytic ehrlichiosis, was also identified as a human disease agent in US, mostly in immunocompromised patients (Buller *et al.*, 1999).

HME and HGA are now considered the most important human diseases caused by Anaplasmataceae members. In the US, 1,223 cases were reported by 30 state health departments from 1986 to 1997, 742 (60.7%) categorized as HME, 449 (36.7%) as HGA and 32 (2.6%) not ascribed to a specific agent (McQuiston *et al.*, 1999; Paddock & Childs, 2003). In 1999, both diseases had become nationally notifiable and official reports show that HGA is more frequently recognized than HME (CDC, 2001, 2002, 2003, 2004, 2005, 2006). As an example, the average reported annual incidences during 2001-2002 were 14 and 6 cases per $10^5$ inhabitants for HGA and HME, respectively (Demma *et al.*, 2005). Moreover, worldwide investigation has revealed increasing evidence of a potential role of *E. chaffeensis* and especially *A. phagocytophilum* in human disease also outside the US. Serological studies have suggested the occurrence of HME in febrile illness patients from Europe (David de Morais *et al.*, 1991; Pierard *et al.*, 1995; Nutti *et al.*, 1998), Africa (Uhaa *et al.*, 1992), and South America (Da Costa *et al.*, 2006), but so far neither *E. chaffeensis* isolation nor DNA detection has been achieved in human cases to reinforce these data. Concerning HGA, the first patient with confirmed disease outside the US was reported from Slovenia in 1997 (Petrovec *et al.*, 1997) and more than fifty cases have been reported in Europe since then. The majority occurred in Northern and Central Europe, especially Slovenia (Lotric-Furlan *et al.*, 2006), but there are also individual case reports from other European countries, later discussed in detail. Polymerase chain reaction (PCR)-based studies of potential tick vectors and reservoir animals have also shown the wide distribution of *A. phagocytophilum* across Europe and in some parts of Middle East and Asia, which will be object of further revision.

It seems that human *A. phagocytophilum*-associated infections rather than *E. chaffeensis* are more commonly recognized over various continents. Thus, HGA possibly represents a widespread and significant public health problem of increasing but undefined magnitude in most countries, and that should be better investigated.

TABLE 2. Biological, ecological and epidemiological features of medically important Anaplasmataceae species.

| Genus/Species | Vector/ Transmission | Host | Target Cell | Disease | Geographic Distribution | First Reference |
|---|---|---|---|---|---|---|
| *Anaplasma* | | | | | | |
| *A. bovis* | Ixodid ticks/ Bite | Ruminants | Monocytes | Bovine "ehrlichiosis" | Africa, Middle East and Asia | Donatien & Lestoquard, 1936 |
| *A. caudatum* | Ixodid ticks / Bite | Ruminants | Erythrocytes | Anaplasmosis | Worldwide | Ristic & Kreier,1984 |
| *A. centrale* | Ixodid ticks / Bite | Ruminants | Erythrocytes | Anaplasmosis | Worldwide | Theiler, 1911 |
| *A. marginale* | Ixodid ticks / Bite | Ruminants | Erythrocytes | Anaplasmosis | Worldwide | Theiler, 1910 |
| *A. ovis* | Ixodid ticks / Bite | Ruminants | Monocytes | | Asia | Lestoquard, 1924 |
| *A. platys* | Ixodid ticks / Bite | Canids | Platelets | Infectious cyclic thrombocytopenia | Worldwide | Harvey *et al.*, 1978 |
| *A. phagocytophilum* | Ixodid ticks / Bite | Ruminants<br>Equines<br>Humans<br>Canines<br>Felines | Neutrophils | Tick-borne fever<br>Equine granulocytic anaplasmosis<br>Human granulocytic anaplasmosis | Europe<br>America and Europe<br>United States and Europe<br>United States and Europe<br>United States and Europe | Gordon *et al.*, 1932<br>Gribble, 1969; Stannard *et al.*, 1969<br>Bakken *et al.*, 1994; Chen *et al.*, 1994<br>Madewell & Gribble, 1982<br>Bjoersdorff *et al.*, 1999c |
| *Aegyptianella* | | | | | | |
| *A. pullorum* | Argasid ticks /Bite | Birds | Erythrocytes | | Worldwide | Carpano, 1928 |
| *Ehrlichia* | | | | | | |
| *E. canis* | Ixodid ticks/Bite | Canines<br>Humans | Monocytes, macrophages | Canine monocytic ehrlichiosis | Worldwide;<br>Venezuela | Donatien & Lestoquard, 1935<br>Perez *et al.*, 1996 |
| *E. chaffeensis* | Ixodid ticks / Bite | Humans<br>Canines | Monocytes, macrophages | Human monocytic ehrlichiosis | United States | Maeda *et al.*, 1987<br>Dawson *et al.*, 1996 |
| *E. ewingii* | Ixodid ticks / Bite | Canines<br>Humans | Neutrophils | Canine granulocytic ehrlichiosis | United States | Ewing *et al.*, 1971<br>Buller *et al.*, 1999 |
| *E. muris* | Ixodid ticks / Bite | Rodents | Monocytes, macrophages | | Japan | Wen *et al.*, 1995 |
| *E. ruminantium* | Ixodid ticks / Bite | Ruminants | Endothelial cells, neutrophils | Heartwater disease | Africa and Caribbean region | Cowdry, 1925 |
| *Neorickettsia* | | | | | | |
| *N. helminthoeca* | Trematodes/ Ingestion | Canines | Monocytes, macrophages | Salmon poisoning disease | United States | Philip *et al.*, 1953 |
| *N. risticii* | Trematodes/ Ingestion | Equines | Monocytes, macrophages | Potomac horse fever | America and Europe | Holland *et al.*, 1985; Rikihisa & Perry, 1985 |
| *N. sennetsu* | Trematodes/ Ingestion | Humans | Monocytes, macrophages | Sennetsu fever | Japan and Malaysia | Misao & Kobayashi, 1955 |

## 1.2. *ANAPLASMA PHAGOCYTOPHILUM*

### 1.2.1. GENERALITIES

*A. phagocytophilum* is a small coccoid to ellipsoidal, often pleomorphic bacterium with a cell diameter ranging from 0.2 to 2.0 µm. Ultrastructurally, it contains a nucleoid with electron-dense DNA strands and ribosomes surrounded by two limiting membranes, an inner cytoplasmic membrane and a rippled outer cell wall. Like the majority of Anaplasmateaceae, this microorganism forms characteristic microcolonies (morulae) in the cytoplasm of infected cells, as the result of the agent's binary fission in a confined area defined by membrane-bounded vacuole (Popov *et al.*, 1998).

The natural history of *A. phagocytophilum* involves both the infection of arthropods, especially *Ixodes* ticks, and several mammalian species, in which man is included as an incidental dead-end host. Depending on the infected host, different cell types are targeted by this agent in a replication cycle that is still not entirely understood. In vertebrates the microorganism has a marked tropism for polymorphonuclear leucocytes, preferentially infecting neutrophils, a characteristic feature often noted with disease. *A. phagocytophilum* causes a pathologic process commonly known as granulocytic anaplasmosis (GA). The designation based on cell tropism may become less useful as additional Anaplasmataceae members are recognized as pathogens and since more than one species may be responsible for the broad category of "granulocytic" disease. However, this nomenclature is firmly established in the literature, and to avoid confusion here it will be used to designate disease caused by *A. phagocytophilum*.

GA affects humans as well as several domestic animals including ruminants, horses, dogs and cats (Gordon *et al.*, 1932; Gribble, 1969; Madewell & Gribble, 1982; Bakken *et al.*, 1994; Chen *et al.*, 1994; Greig *et al.*, 1996; Bjoerdorf *et al.*, 1999c). The disease is generically characterized as a non-specific febrile illness accompanied by hematological abnormalities and hepatic injury. Historically, GA has been recognized by different designations, each of them associated with fingdings thought to be caused by a distinct etiologic agent. According to the affected host and implicated pathogen, the disease was named ruminant tick-borne fever (or pasture fever), equine granulocytic ehrlichiosis and human granulocytic ehrlichiosis as the result of infections by *E. phagocytophila*, *E. equi* and the HGE agent, respectively. The unification of these agents into a

single species proposed by Dumler and coworkers (2001) expanded the dimensions and broadened the perspective of this tick-borne disease.

Not cultivable in cell-free media or chicken embryos, the *in vitro* manipulation of *A. phagocytophilum* was initially based on direct purification from infected blood or short-term cultures of blood cells collected from infected animals. The development of cell culture systems able to mimic *in vivo* situations made the continuous propagation of the pathogen possible and allowed a more detailed analysis of its biological features. The first *in vitro* isolation of *A. phagocytophilum* was achieved by Goodman and coworkers (1996) after inoculation of a patient's blood in a human promyelocytic leukemia cell line (HL-60, ATCC CCL-240) developed by Gallagher and coworkers (1979). In addition to undifferentiated cell lines, other cells are also currently used for *A. phagocytophilum* cultivation, including HL-60 cells induced to differentiate into functional granulocytes (rHL-60), as well as embryonic *Ixodes scapularis* tick cell lines IDE8 and ISE6 (Goodman *et al.*, 1996; Munderloh *et al.*, 1996, 1999; Heimer *et al.*, 1997). Furthermore, the development of murine models that in part reproduced the infection process has greatly expanded the understanding of *A. phagocytophilum* pathogenesis. The use of inbred and targeted gene-deleted (knockout) mice has allowed insight into precise mechanisms of disease and host cell-pathogen interactions in the context of a whole organism, complemented studies based on *in vitro* tissue culture, *ex vivo* human and ruminant neutrophil cultures and *in vivo* large animal experiments (Borjesson & Barthold, 2002).

The complete genome sequence of *A. phagocytophilum* HZ strain has been recently achieved by Hotopp and coworkers (2006), illustrating the existence of a single circular chromosome of 1,471,282 bp with an average G+C content of 41%. Comparison analysis has shown that most genomic features of *A. phagocytophilum* are typical of other sequenced Anaplasmataceae, but also reveals new species-specific genes indicating particular niche adaptations (Hotopp *et al.*, 2006).

### 1.2.2. LIFE-CYCLE

The study of naturally- and experimentally-infected neutrophils as well as human and tick-derived cell cultures has allowed a detailed analysis of the intracellular replication cycle of *A. phagocytophilum* (Chen *et al.*, 1994; Goodman *et al.*, 1996; Munderloh *et al.*, 1996, 1999;

Popov *et al.*, 1998; Webster *et al.*, 1998). This bacterium undergoes a simple cycle involving three fundamental steps: invasion of host cells likely through receptor-mediated endocytosis; proliferation by binary fission in a membrane vacuole that does not fuse with lysosomes (parasitophorous vacuoles); exit via rupture of both *A. phagocytophilum*-containing vacuole and the adjacent host cell membrane when the morula is located at the periphery of the infected cell.

In general, morulae tend to be much larger and contain more microorganisms in tick cells than in neutrophils or HL-60 cells. Pathogen morphology is also more variable presenting a higher degree of pleomorphism (Munderloh *et al.*, 1996, 1999). Genome analysis has shown that *A. phagocytophilum* lacks genes for both lipopolysaccharide and peptidoglycan biosynthesis (Lin & Rikihisa, 2003; Hotopp *et al.*, 2006). The peptiglycan layer is a polymer consisting of sugars and amino acids that form a homogeneous layer between the plasma membrane and the cell-wall of eubacteria, conferring structural strength to bacteria. The lack of this layer justifies the morphologic pleomorphism of *A. phagocytophilum* that should be expected to be higher in environments with less osmotic pressure than those experienced inside the body of invertebrate vector (Rikihisa, 2006b).

In both mammalian and tick cells, *A. phagocytophilum* presents two distinct ultrastructural forms: reticulate cells (RC) characterized by a loosely packed and randomly dispersed nucleoid and dense-cored cells (DC) with a condensed electron-dense protoplast. By temporal examination of *A. phagocytophilum* development in HL-60 cells, Webster and coworkers (1998) concluded that the early forms of the agent have the round reticular appearance while later structures are small and dense. In tick-cells the agent also presented the same temporal pattern; *A. phagocytophilum* colonies comprise appreciable numbers of condensing organisms in older tick-cell cultures (Munderloh *et al.*, 1996, 1999). This data suggests the existence of an intracellular biphasic development that undergoes from RC towards DC cells, similar to that observed for other closely related agents such as *A. marginale* (Blouin & Kocan, 1998) and *E. chaffeensis* (Zhang *et al.*, 2007), in which DC are the infective form and RC the metabolic active form that multiplies by binary fission (Figure 3). However, both the dense core and reticulate forms undergo binary fission *in vitro*, suggesting plasticity to any potential developmental or differentiation cycle (Popov *et al.*, 1998).

Depending on the infected host, *A. phagocytophilum* replication takes place in different cells types. In naturally infected ticks the agent has been detected in midgut cells, hemocytes and salivary gland cells (Magnarelli *et al.*, 1995b; Telford *et al.*, 1996; Alberti *et al.*, 1998; 2000;

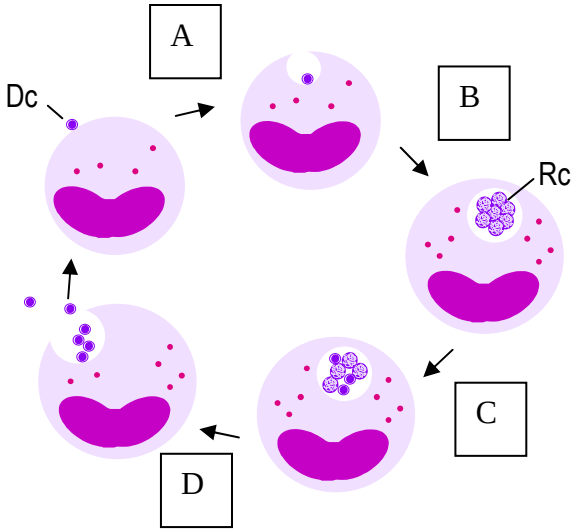



FIGURE 3. Representation of the intracellular cycle proposed for *A. phagocytophilum*.

A- Dense core (Dc) microorganism starts the cycle by attaching and entering into a susceptible host cell; B- Once inside the cell it remains within the vacuole that does not fuse with lysossomes. The microorganism differentiates into a metabolic active reticulated form (Rc) and multiplies by binary fission, forming characteristic dense packed microcolonies known as morulae; C- After multiple rounds of division Rc starts to differentiate into DC forms; D- Finally, by exocytosis or host cell lysis the infectious DC are released to initiate new cycles in new host cells (Blouin & Kocan, 1998; Zhang *et al.*, 2007).

Kim *et al.*, 2003; Ohashi *et al.*, 2005). These different locations are presumed to represent part of a life-cycle similar to that observed for *A. marginale* (Ge *et al.*, 1996; Kocan *et al.*, 2004). It is assumed that following ingestion in the blood meal by feeding ticks, *A. phagocytophilum* primarily infects midgut epithelial cells, passes through this barrier and, via circulating hemocytes, subsequently colonizes salivary gland cells from where it is transmitted to vertebrates via salivary emissions released during the next feeding. The infection of salivary glands seems to be an early event that may take place during or soon after the infective blood meal, since questing ticks already present *A. phagocytophilum* in acinus cells (Telford *et al.*, 1996; Ohashi *et al.*, 2005). Up to now, salivary glands are regarded as the most important organ for the agent's maintenance in vector ticks and subsequent transmission to vertebrate hosts. Other organs might also be infected by *A. phagocytophilum*, which may contribute to the agent's ability to persist in the vector tick, but it does not seem to include ovarian tissue. In fact, infected ticks show persistent bacteremia, with the agent surviving arthropod molts and being maintained in successive life stages (transstadial transmission) but with no, or inefficient offspring infection (transovarial transmission) (Richter *et al.*, 1996; Telford *et al.*, 1996; Des Vignes & Fish 1997; Hodzic *et al.*, 1998; Goethert & Telford, 2003). Of interest, Sukumaran and coworkers (2006) described an *Ixodes* tick salivary gland protein, Salp16, that in part determines successful colonization of salivary glands by *A. phagocytophilum* after acquisition of a blood meal from an infected mammalian host.

Once inside a mammalian host the agent targets cells derived from bone marrow precursors, most often neutrophils and band neutrophils. The infection is not localized, affecting circulating peripheral blood cells, but *A. phagocytophilum* has occasionally been detected in

spleen, lung, liver, bone marrow, lymph node and heart (Madigan *et al.*, 1995; Walker & Dumler, 1997; Bunnel *et al.*, 1999; Lepidi *et al.*, 2000; Martin *et al.*, 2000, 2001; Trofe *et al.*, 2001; Remy *et al.*, 2003; Bayard-Mc Neeley *et al.*, 2004). Both *ex vivo* and *in vitro* data have emphasized that pathogen tropism is especially directed to the mature granulocyte population (Heimer *et al.*, 1997; Klein *et al.*, 1998; Zeman *et al.*, 2002a; Bayard-Mc Neeley *et al.*, 2004) and its detection in several organs seems to reflect vascular perfusion or focal sequestration of infected neutrophils (Lepidi *et al.*, 2000). However, Walker and Dumler (1997) also discussed the possibility that the ligand recognized by *A. phagocytophilum* adhesion is broadly distributed over a wide variety of cells from different lineages, justifying the occasional detection of this agent in endothelial cells, fibroblasts and macrophages. Recent experimental studies have reinforced the hypothesis that other cell types might be involved in *A. phagocytophilum* cycle by the demonstration that the bacterium is capable of infecting endothelial cells and passing from the infected endothelium into neutrophils (Munderloh *et al.*, 2004; Herron *et al.*, 2005). As conjectured in those studies an endothelial reservoir cell may mediate the infection of blood cells and *A. phagocytophilum* spreads from the site of tick feeding, and offers opportunities for ongoing, direct cell-to-cell infection of neutrophils, avoidance of host immune effectors, and completion of the pathogen life-cycle by infection of circulating leukocytes available for transfer to blood-feeding ticks (Munderloh *et al.*, 2004; Herron *et al.*, 2005).

### 1.2.3. PATHOGENESIS

*A. phagocytophilum* undergoes substantial selection pressure to be able to survive and replicate in different environments during its life-cycle. In vector ticks, pathogens must survive extreme fluctuations in temperature, pH, osmotic pressure, and other factors related to the physiological status of these arthropods. In vertebrate hosts, they must overcome the highly developed inflammatory and immunologic defences of mammals. Moreover, A. *phagocytophilum* possesses an unusual intracellular lifestyle in that it colonizes neutrophils, the key cells in innate immunity and the first line of defence against invading pathogens. These short-lived, terminally differentiated cells ingest invading microorganisms and destroy them by various means, which include fusing the bacteria-containing phagosome with acidic lysosomes as well as directing toxic oxidative and proteolytic compounds into the phagosomal lumen (Mayer-Scholl *et al.*, 2004). The

specific cell tropism displayed by *A. phagocytophilum* is thus far known to be shared only by *E. ruminantium*, *E. ewingii*, and *Chlamydophila pneumoniae*, among eubacteria, and indicates that these microorganisms have developed strategies to evade and/or subvert neutrophil killing mechanisms in order to promote their survival and perpetuation. Recent investigations have been providing important data regarding *A. phagocytophilum* survival strategies and interactions with their host-cells, allowing a more complete understanding of granulocytic anaplasmosis pathogenesis (Borjesson & Barthold, 2002; Carlyon & Fikrig, 2003, 2006; Dumler, 2005; Dumler *et al.*, 2005a).

Lack of lipopolysaccharide

An adaptation of *A. phagocytophilum* that likely increases its chance of successful colonization of vertebrate neutrophils is the lack of lipid A and peptidoglycan biosynthesis genes (Lin & Rikihisa, 2003). Lipopolysaccharide (LPS) elicits profound innate immune responses via interactions with Toll-like receptor 4 and activating leukocytes and proinflammatory responses. Therefore, it is interesting to that comparative genomics among the Anaplasmataceae reveals cell-wall biosynthetic machinery only in *W. pipientis* and *A. marginale*, neither of which are associated with leukocyte infection (Hotopp *et al.*, 2006). Thus, LPS genes could have either been horizontally acquired in these microorganisms or were lost in the other Anaplasmataceae as an adaptation to successfully infect vertebrate immune cells (Hotopp *et al.*, 2006).

Antigenic variation

*A. phagocytophilum* has a genome with numerous repeats of functionally important genes, especially those of surface proteins. The whole genome sequence of *A. phagocytophilum* HZ strain demonstrates the existence of three outer membrane protein (*omp*)-1, one major surface protein (*msp*)-2, two *msp*2 homologs, one *msp*4, and one hundred and thirteen 44-kDda surface protein(*p44*) loci belonging to the Omp-1/Msp2/P44 superfamily (Hotopp *et al.*, 2006)[2]. The *p44*s represent the largest expansion of this surface protein superfamily and are annotated as full-length silent/reserve, truncated, and fragments that recombine to generate antigenic variation (Figure 4). The expression of as many as 65 different *p44* genes (p44–1 to p44–65) has been reported in infected mammals and ticks (Felek *et al.*, 2004; Lin *et al.*, 2002; Wang *et al.*, 2002;

[2] Although established in the literature, the Omp-1/Msp2/P44 division is controversial, and some researchers defende that insufficient differences exist in the surface proteins to warrant the division, thus referring to them by the generic designation of Msp2.

Zhi *et al.*,2002). Additionally, genome sequencing of *A. phagocytophilum* HZ strain has identified 23 novel *p44* genes (p44-66 to p44–88), but they have not yet been experimentally identified as being expressed (Hotopp *et al.*, 2006).

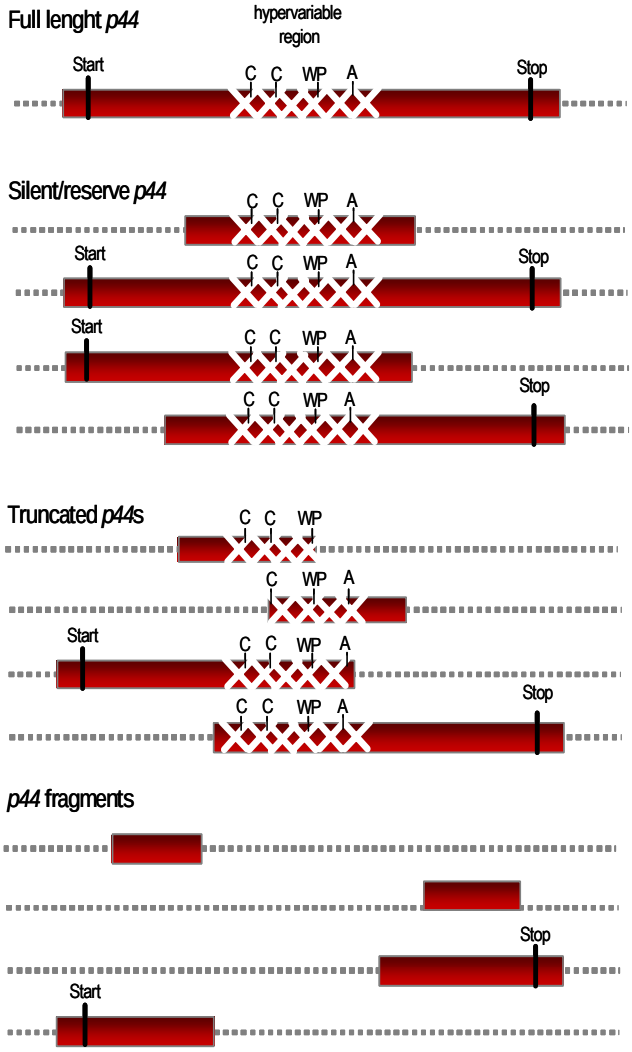



FIGURE 4. Representation of *p44* genes (adapted from Hotopp *et al.*, 2006).

The *p44*s consist of a central hypervariable region of approximately 280 bp containing a signature of four conserved amino acid regions (C, C, WP, A) and conserved flanking sequences longer than 50 bp. In *A phagocytophilum* HZ strain there are 22 full-length *p44*s identified that have ORFs longer that 1.0 Kb with conserved start and stop codons. These genes recombine into one of three genomic expression loci (p44ES/APH_1221) [(Barbet *et al.*, 2003; Lin *et al.*, 2003) in Hotopp *et al.*, 2006]. Other 64 shorter *p44*s were identified presenting ORFs less than 1.0 Kb and likely serve as reserve/silent *p44*s. They may have either the conserved or alternative start and/or stop codons and are not likely to be expressed at their current genome location, but can recombine into the expression locus (p44ES/APH_1221). In addition 21 5' and 3' fragments and 6 truncations of *p44* genes larger than 60 nucleotides have been identified in the genome. Truncated *p44*s carry the complete hypervariable region, or a portion thereof, but only one of the two conserved regions. Fragments of *p44* have only a conserved region and no hypervariable region. Each annotated *p44* is longer than 60 bp, although smaller fragments can be identified throughout the genome. These, as well as *p44* truncations and fragments, are likely to be non functional remnants of previous recombination events.

Antigenic variation of surface proteins is regarded as a mechanism for promoting the pathogen's evasion of the immune system and persistence in the vertebrate host. This strategy is evident in other arthropod-borne agents such the extracellular *Borrelia hermsii* and *Trypanosoma brucei*, but also in the closely related intracellular *A. marginale* (Barbour & Restrepo, 2000 *in* Palmer *et al.*, 2006). Like those pathogens, *A. phagocytophilum* cannot be transovarially inherited in its arthropod vector and its persistence in vertebrates may be a mechanism for promoting

pathogen acquisition by ticks and transmission to new hosts. As surface proteins of *A. phagocytophilum* has been demonstrated to be adhesins, antigenic variation may also represent an ecological adaptation to different host environments or varying species host cells independent of immune selection. As showed by Zhi and coworkers (2002), transcription of specific *p44*s is dominantly expressed by *A. phagocytophilum* in mammals at an early stage of infection and in HL-60 cells at 37ºC but differ from those associated to ticks that are upregulated in infected HL-60 cell at low temperatures of 24 to 25ºC (Zhi *et al.*, 2002). Restricted transcription and expression of *p44* over many passages in cell culture and in tick salivary gland cells suggest selection by fitness for new niche, a finding underscored by the role of surface proteins to act as adhesins (Zhi *et al.*, 2002; Scorpio *et al.*, 2004a; Park *et al.*, 2003a; Dumler *et al.*, 2005a). However, more recent comprehensive studies do not confirm a restricted expression profile *in vitro* or *in vivo*, suggesting the concept that the changing expression of surface proteins does little to promote survival via antigenic variation among animals with a self-limited infection (Choi *et al.*, 2007).

Cell invasion by mediated-endocytosis and isolation of the parasitophoros vacuole

It has been suggested that *A. phagocytophilum* attaches to neutrophils by specific interaction between surface proteins and fucosylated, and possibly sialylated ligands, of surface proteins; platelet selectin glycoprotein ligand-1 (PSGL-1) has been chiefly implicated in this process (Goodman *et al.*, 1999; Herron *et al.*, 2000, Park *et al.*, 2003a). Such a receptor-mediated interaction seems to be determinant in *A. phagocytophilum* tropism for neutrophils and leads to the downstream events resulting in pathogen internalization by endocytosis and successful colonization of host cells (Mott *et al.*, 1999; Carlyon & Fikrig, 2003, 2006).

One of the primary means by which neutrophils destroy phagocytosed pathogens is through the production of toxic oxygen intermediates derived from superoxide anion ($O_2^-$). The pH change resulting from the $O_2^-$ influx triggers a potassium-dependent release of proteases which along with reactive oxygen species (ROS) kill and degrade ingested microorganisms (Mayer-Scholl *et al.*, 2004). $O_2^-$ is produced by the multi-component enzyme, NADPH oxidase that lies unassembled in resting cells and is activated by several stimuli, such as bacterial uptake (Figure 5). It has been shown that the addition of *A. phagocytophilum* to neutrophils elicits little or no detection of ROS *in vitro* (Mott & Rikihisa, 2000; Carlyon *et al.*, 2004; Ijdo & Mueller, 2004 Borjesson *et al.*, 2005). This likely stems from the fact that the pathogen enters its host-cell via non-opsonin phagocytic receptor-mediated endocytosis (Mott *et al.*, 1999), which presumably

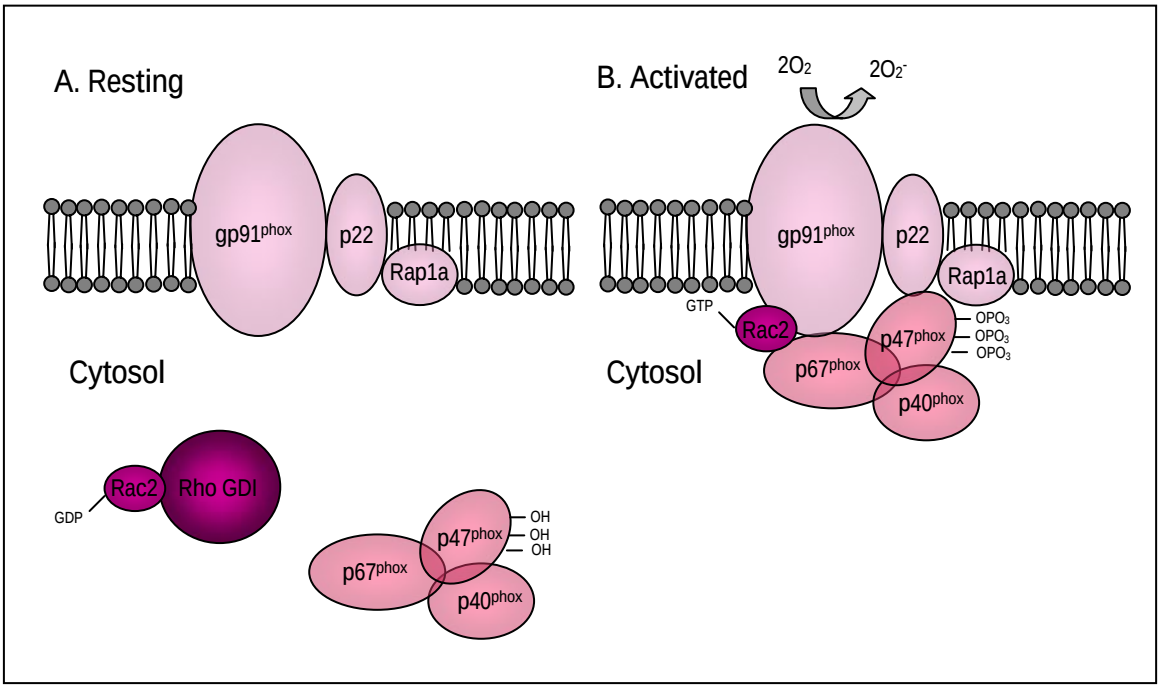



FIGURE 5. Representation of NADPH oxidase (adapted from Carlyon & Fikrig, 2003, 2006).

NADPH oxidase is a multi-component enzyme that lies unassembled in resting neutrophils (A). The cytochrome $b_{558}$, consisting of gp91$^{phox}$ and p22$^{phox}$, is integrated into the membrane of secretory vesicles and specific granules. The additional subunits are present in the cytosol, and include p40$^{phox}$, p47$^{phox}$, p67$^{phox}$ and Rac2. This latter component functions as the molecular switch for initiating the respiratory burst. Upon activation, either by the binding of opsonized microorganisms or by binding of high concentrations of chemoattractants to their cognate surface receptors, Rac2 dissociates from its inhibitor, RhoGDI, switches from a GDP- to a GTP-bound state and migrates to the site of oxidase assembly. The vesicle/granules fuse with the phagosomal or cell membranes to deliver cytochrome $b_{558}$ to the site of oxidative formation. Concomitantly, p47phox becomes phosphorylated and, along with the other cytosolic components translocate to join cytrochrome $b_{558}$ and form the functional enzyme (B). The redox center of assembled oxidase transfer electrons from NADPH to molecular oxygen to generate $O_2^-$ outside the cell or within the lumen containing the ingested microorganism.

allows it to bypass the phagocytic route of oxidative stimulation. However, several studies have demonstrated that *A. phagocytophilum*-neutrophils interaction results in a dose-dependent stimulation of NADPH oxidase assembly and degranulation (Choi & Dumler, 2003; Carlyon *et al.*, 2004; Choi *et al.*, 2004). This suggests that the pathogen is able to directly detoxify $O_2^-$, providing it with a means of protection from oxidative damage and contributing to the inability to detect ROS *in vitro* (Carlyon *et al.*, 2004; Borjesson *et al.*, 2005). Although the $O_2^-$ scavenging mechanism is currently undefined, genomic location and transcriptional expression of a superoxide dismutase homologue (*sodB*) has been reported for *A. phagocytophilum* (Ohashi *et al.*, 2002). Superoxide dismutase is an enzyme that catalyses the rapid dismutation of $O_2^-$ to $H_2O_2$ and $O_2$. The cotranscription of *sod*B with components of the type IV secretion system may also facilitate the transfer of molecules between the *A. phagocytophilum* and the host (Ohashi *et al.*, 2002;

Hotopp *et.al.*, 2006). Moreover, genome sequencing has identified two other ortholog clusters of proteins potentially involved in response to oxidative stress - a putative heme copper oxidase and a putative flavohemoglobin (Hotopp *et al.*, 2006).

After entering the host cell, *A. phagocytophilum* resides in an endosome that ceases to mature and does not fuse with lysosomes. The nature of this parasitophorous vacuole is unique when compared with other vacuoles resulting from either phagocytosis or endocytosis. Several studies have showed that although *A. phagocytophilum* vacuoles are part of the endocytic pathway, they are somehow modified and do not colocalize with markers of either early or late endosomes (Webster *et al.*, 1998; Gokce *et al.*, 1999; Mott *et al.*, 1999). The vacuolar membrane also fails to colocalize with cytochrome b558, demonstrating that *A. phagocytophilum* continues to overcome the oxidative killing capacity by inhibiting the fusion of cytochrome b558-carrying vesicles/granules (Carlyon *et al.*, 2004; Ijdo & Mueller, 2004). It thus seems that *A. phagocytophilum* avoids destruction by isolating itself from host endocytic and exocytic vesicular traffic (Rikihisa, 2003). This phenomenon is restricted to *A. phagocytophilum*-containing vacuoles and is dependent on bacterial protein synthesis, as oxytetracycline treatment results in vacuole maturation into phagolysosomes (Gokce *et al.*, 1999; Ijdo & Mueller, 2004).

Subversion of host cell functions

Besides evading the host cell killing mechanisms as above mentioned, *A. phagocytophilum* subverts several biological functions of its host cell and thereby potentially promotes its intracellular survival and dissemination.

One of the most critical subversions elicited by *A. phagocytophilum* is the inhibition of NADPH oxidase activity in their host cells. Although not yet shown in neutrophils, HL-60 cells with established infections become severely inhibited in their ability to generate reactive oxygen species (ROSs) (Banerjee *et al.*, 2000; Wang *et al.*, 2002). This phenomenon is time-dependent and has been linked to the inhibition of transcription of genes encoding two NADPH oxidase components, $gp91^{phox}$ and Rac2 (Banerjee *et al.*, 2000; Carlyon *et al.*, 2002; Choi & Dumler, 2003). The mechanism by which the pathogen downregulates *CYBB* expression (gene encoding $gp91^{phox}$) has been already identified in HL-60 cells and is related to the inhibition of promoter activity (Thomas *et al.*, 2005). However the pathogen's determinants implicated in the process are currently unknown. An interesting observation regards the localization of *A. phagocytophilum* AnkA, a 153-160 kDa protein with at least 11 tandemly repeated ankyrin motifs with no homology with other proteins

(Caturegli *et al.*, 2000). Park and coworkers (2004) showed that this protein forms a complex with the chromatin of infected HL-60, binding to nuclear proteins and complexes to AT-rich nuclear DNA that lacks specific conserved sequences. As mentioned by Dumler and coworkers (2005b), the mere presence of AnkA in the nucleus of a cell in which gene transcription appears to be altered by infection compels further investigation of a direct pathogenesis role in regulation of eukaryotic gene expression.

Delayed apoptosis is another abnormal function of *A. phagocytophilum* infected neutrophils. Apoptosis is a process that regulates inflammation by programmed cell death of activated neutrophils usually within 24 to 48 hours (Dumler *et al.*, 2005a). *A. phagocytophilum* not only fails to activate the apoptosis program usually triggered by bacterial uptake, but it also delays spontaneous neutrophil programmed cell death by dysregulating and delaying the expression of apoptosis-associated genes (Yoshiie *et al.*, 2000; Scaife *et al.*, 2003; Borjesson *et al.*, 2005; Ge *et al.*, 2005). This phenomenon is not dependent on viable bacteria and is mediated, at least in part, by a heat-resistant surface molecule, as heat-killed *A. phagocytophilum* delays apoptosis as effectively as live bacteria (Borjesson *et al.*, 2005).

Additional dysregulations of neutrophil function by *A. phagocytophilum* includes reduction of adhesion to endothelial cells and inhibition of phagocytosis (Choi *et al.*, 2003, 2004; Garyu *et al.*, 2005). The adhesion defects have been associated with shedding of PSGL-1 and L-selectin as a result of cell degranulation (Choi *et al.*, 2003, 2004). The inhibition of phagocytosis seems to results from alterations of Rac2 expression and loss of important surface receptors (Garyu *et al.*, 2005).

Altogether the activated-deactivated phenotype of the *A. phagocytophilum*-infected neutrophil may benefit the bacterium by increasing concentrations of infected cells in the peripheral blood that are unresponsive to tissue recruitment and may have a prolonged lifespan. However, the cost to the host includes activation of neutrophils to participate in proinflammatory reactions while they are unable to act as microbicidal effectors or regulators of inflammation (Dumler *et al.*, 2005a). It has long been speculated that the antimicrobial capacities of granulocytes infected with *A. phagocytophilum* was altered, in large part this was due to early recognition that the pathogen in sheep and cattle exacerbates other diseases, including louping ill, listeriosis, pasteurellosis, staphylococcal abscesses, and parainfluenza 3 [(Gordon *et al.*, 1932; Foggie, 1951, 1956; Gronstol & Ulvund, 1977; Gilmour *et al.*, 1982; Batungbacal & Scott, 1982) *in* Borjesson & Barthold, 2002; Stuen *et al.*, 2003b]. Severe opportunistic infections have also been associated to fatal cases of

HGA, including invasive pulmonary aspergillosis, cryptococcal pneumonia, ulcerative *Candida* and herpes simplex esophagitis (Bakken *et al.*, 1994; 1996c; Hardalo *et al.*, 1995; Walker & Dumler, 1997; Jahangir *et al.*, 1998; Lepidi *et al.*, 2000). Although not directly proven, it is likely that loss of one of the primary means of host-mediated microbial killing contributes to the increased susceptibility to other infections (Carlyon & Fikrig, 2003).

Modulation of the inflammatory response

Two independent investigations have shown that neutrophils, bone marrow progenitors or HL-60 cells differentiated into neutrophil-like cells (rHL-60) infected with *A. phagocytophilum* produce striking quantities of CXC and CC chemokines, including monocyte chemotactic protein (MCP)-1, macrophage inflammatory protein (MIF)-1$\alpha$, MIF-1 $\beta$, regulated on activation normal T-cell expressed and secreted (RANTES) and interleukin (IL)-8, but not the classic pro-inflammatory cytokines IL-1, IL-6 and tumor necrosis factor (TNF)-$\alpha$ (Klein *et al.*, 2000; Akkoyunlu *et al.*, 2001). Chemokine production is induced specifically by a heat-stable *A. phagocytophilum* component independent of classic LPS stimulation (Klein *et al.*, 2000), suggesting a potential role for the pathogen in neutrophil activation. In fact, Akkoyunlu and coworkers (2001) dissected the biological relevance of chemokine production, especially the neutrophil chemoattractant IL-8, during *A. phagocytophilum* infection. They showed that bacterium induces IL-8 secretion from rHL-60 cells in a dose- and time-dependent fashion and the incubation with supernatants from *A. phagocytophilum*-infected, but not uninfected cells, results in chemotaxis of human neutrophils similar to that of recombinant IL-8. Human neutrophils respond to IL-8 through the receptors CXCR1 and CXCR2 (Mahalingam & Karupiah, 1999 *in* Carlyon & Fikrig, 2003). It was also showed that *A. phagocytophilum*-infected rHL-60 cells demonstrate elevated surface expression of CXCR2, and the antibody blockade of this receptor resulted in a decreased bacteremia (Akkoyunlu *et al.*, 2001). Moreover, *in vivo* experiments also showed the importance of neutrophil chemoattraction in *A. phagocytophilum* infection. Blockade of the murine homolog CXCR2 results in considerable decreases in bacterial load compared with control background (Akkoyunlu *et al.*, 2001; Scorpio *et al.*, 2004b). As an obligate intracellular bacterium, the successful maintenance of *A. phagocytophilum* is dependent on its transfer to new host-cells, thus inciting chemokine production could be a mechanism of attracting naïve neutrophils to sites of infection for further bacterial propagation (Carlyon & Filrig, 2003).

The disadvantage of inciting chemokine production to the host is that recruitment of inflammatory cells that are activated and could induce inflammation, leads to damage to tissues (Dumler *et al.*, 2005a). Sera from HGA patients (Dumler *et al.*, 2000) and experimentally inoculated mice (Akkoyunlu & Fikrig, 2000; Martin *et al.*, 2000) demonstrate elevated levels of interferon (IFN)-$\gamma$ and IL-10, but lack TNF-$\alpha$, IL-1$\beta$, IL-4 and IL-6, which suggests a role for macrophage activation in recovery and disease (Dumler *et al.*, 2005a). It is well established that IFN-$\gamma$ protects against infections by obligate intracellular bacteria and against *A. phagocytophilum* (Akkoyunlu & Fikrig, 2000; Martin *et al.*, 2001). However, there is a consequence to such protection as IFN-$\gamma$ is also damaging against host cells (Fresno *et al.*, 1997 *in* Carlyon & Fikrig, 2003). Kinetic studies of the distribution of *A. phagocytophilum*-infected neutrophils and inflammatory injury in mouse models suggested a role of IFN-γ in restriction of infection but also in tissue injury (Martin *et al.*, 2000, 2001). As demonstrated by Martin and coworkers (2001) *A. phagocytophilum* IFN-γ knockout mice have a 6- to 7-fold increase in bacteremia compared to wild-type controls but with no histopathologic lesions. In contrast, IL-10 knockout mice, which poorly restrict IFN-γ production, present an identical bacterial load to that of wild-type mice although a substantially greater degree of inflammatory histopathologic injury (Martin *et al.*, 2001). Several other murine models have showed that host innate immune response plays a more important role in histopathologic lesions than does pathogen load. Indeed, the infection of mice in which innate immunity has been genetically altered, such as Toll-like receptor 2 (TLR2)-, Toll-like receptor 4 (TLR4)-, TLR adaptor molecule MyD88, TNF-α and *CYBB* knockout mice does not affect bacterial burden, yet abrogates inflammatory tissue lesions (Von Loewenich *et al.*, 2004; Scorpio *et al.*, 2005). Such findings suggest that disease relates to immune effectors that inadvertently damage tissue. Indeed, in HGA tissue histopathology and clinical illness are greater than predicted by bacterial burden (Walker & Dumler, 1997; Dumler & Bakken, 1998; Lepidi *et al.*, 2000). The disease processes appear to be immune and inflammatory in nature, not directly related to pathogen burden, and the result of triggering of a detrimental and poorly regulated host response (Dumler, 2005).

*A. phagocytophilum* modulation of host factors in order to promote survival and dissemination might not be limited only to vertebrates. A recent investigation performed by Sukumaran and coworkers (2006) has shown that the pathogen interacts with its vector tick modulating the gene expression of salivary proteins (Salps). In a comprehensive series of experiments, the authors measured the production of 14 Salps and documented a 55-fold

increase in Salp16 expression in salivary gland tissue of *I. scapularis* nymphal ticks infected with *A. phagocytophilum*. Production of ten other salivary proteins was not affected, whereas Salp10, Salp13 and Salp17 were downregulated (Sukumaran *et al.*, 2006). The study also showed that the inhibition of Salp16 affects pathogen survival in midgut and successful colonization of arthropod salivary glands (Sukumaran *et al.*, 2006). However, the mechanism by which *A. phagocytophilum* modulates Salp expression, the direct role of Salp16, or other salivary proteins in pathogen survival, or the consequences for the vector tick is so far unknown. The molecular aspects of arthropod–pathogen interactions are only beginning to be explored.

### 1.2.4. ECOLOGY

*A. phagocytophilum* has a circumglobal distribution within the Northern hemisphere, being maintained in enzootic cycles that correlate with the geographic distribution of vector ticks and competent reservoir hosts. Ticks from the *Ixodes persulcatus*/*ricinus* genospecies complex Oliver & Needham, 1992 (Camicas *et al.*, 1998), are considered the primary vectors for this agent, comprising *I. scapularis* and *I. pacificus*, respectively in Eastern and Western parts of North America, *I. ricinus* in Western, Central and Northern Europe (Macleod & Gordon, 1933; Richter *et al.*, 1996; Telford *et al.*, 1996) and most certainly *I. persulcatus* in Western Europe and Asia (Cao *et al.*, 2000, 2003, 2006; Morozova *et al.*, 2002; Kim *et al.*, 2003, 2006; Wen *et al.*, 2003; Rar *et al.*, 2005) (Figure 6). Other species of the genus *Ixodes* are also involved in perpetuation of *A. phagocytophilum* cycles, such as *I. spinipalpis* in Colorado (Zeidner *et al.*, 2000; Burkot *et al.*, 2001), *I. dentatus* in New England Coast (Goethert & Telford, 2003), and possibly *I. trianguliceps* in United Kingdom (Ogden *et al.*, 1998; Bown *et al.*, 2003). Although the direct role of these latter ticks in human and domestic animal disease is discussed due to their relative host specificity and limited questing potential, when compared with members of *I. persulcatus/ricinus* complex, they are regarded as important bridge-elements, maintaining parallel transmission cycles that would provide support for primary vector infections (Zeidner *et al.*, 2000; Bown *et al.*, 2003; Goethert & Telford, 2003). Additionally, *A. phagocytophilum* has also been detected in the salivary glands of questing *I. ovatus* in Japan, but the vector competence of this species still needs to be proven (Ohashi *et al.*, 2005). The same is true for almost all other non-*Ixodes* ticks and mites that have been sporadically reported with *A. phagocytophilum* DNA, including *Dermacentor variabilis*, *Dermacentor occidentalis*

and *Haemaphysalis leporispalutris* in US (Goethert & Telford, 2003; Holden *et al.*, 2003), *Haemaphysalis longicornis* in Korea (Kim *et al.*, 2003), *Hyalomma detritum* in Tunisia (Sarih *et al.*, 2005), *Dermacentor silvarum* in China (Cao *et al.*, 2006), the Trombiculidae mite *Neotrombicula autumnalis* in Spain (Fernandez-Soto *et al.*, 2001), Syringophilidae quill mites *Torotrogla merulae and Syringophilopsis sturni* in Poland (Skoracki *et al.*, 2006). The majority of these arthropods were collected when feeding in vertebrate hosts. Thus the possible role of host infected blood in *A. phagocytophilum* positive results should not be excluded. Regarding *H. leporispalutris* and *D. variabilis*, experimental studies have already demonstrated that although these ticks are infected by *A. phagocytophilum* they are unable to either maintain the agent transstadially or to successfully transmit it to a susceptible vertebrate host, respectively, and are now considered incompetent vectors (Des Vignes *et al.*, 1999; Goethert & Telford, 2003).

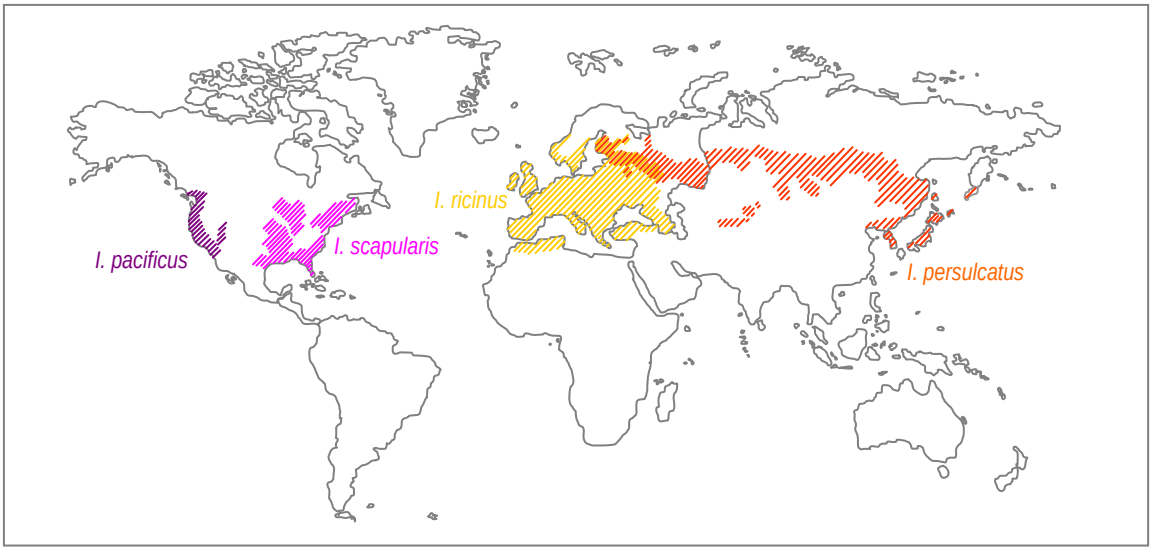



FIGURE 6. Approximate geographic distributions of four medically important *Ixodes persulcatus/ricinus* complex ticks (adapted from Swanson *et al.*, 2006).

Numerous PCR-based studies have accessed the prevalence of *A. phagocytophilum* infection among ticks in several countries. However, they are difficult to compare due to considerable differences in the methods of tick collection, specimen preparation, DNA extraction, selection of nucleic acid probes (primers), and in some cases the lack of further sequencing of positive samples. In general, these studies show pronounced differences according to spatial and temporal distribution, tick species and stage. Table 3 summarizes the prevalences of *A. phagocytophilum* in questing ticks according to different geographic location.

TABLE 3. Prevalence (%) of questing ticks detected with *A. phagocytophilum* infections by PCR-based methods*.

| Region/Tick species | | Comulative Analysis Mean prev (n**) | Median Analysis Median prev | Minimum-maximum prev |
|---|---|---|---|---|
| Africa | | | | |
| | *Ixodes ricinus* | 0.5 (418) | 0.5 | 0.0 – 1.0 |
| Asia | | | | |
| | *Dermacentor silvarum* | 0.7 (286) | 0.7 | |
| | *Ixodes ovatus* | 14.1 (64) | 14.1 | |
| | *Ixodes persulcatus* | 4.3 (1,876) | 4.0 | 0.8 –18.6 |
| Europe | | | | |
| | *Haemaphysalis cocina* | 2.1 (47) | 1.6 | 0.0 – 3.1 |
| | *Ixodes ricinus* | 4.7 (23,741) | 4.3 | 0.0 – 57.1 |
| | *Ixodes persulcatus* | 1.8 (563) | 2.4 | 0.0 – 12.5 |
| North America | | | | |
| Eastern part | *Ixodes scapularis* | 12.5 (5,881) | 11.5 | 0.0 – 50.0 |
| Western part | *Dermacentor occidentalis* | 0.6 (651) | 1 | 0.0 – 6.1 |
| | *Dermacentor variabilis* | 4.4 (113) | 7.6 | 0.0 – 15.2 |
| | *Ixodes pacificus* | 3 (3,934) | 5.2 | 0.0 – 33.3 |

*All the presented data comes from published reports:
African references - Sarih *et al.*, 2005;
Asian references- Cao *et al.*, 2000, 2003, 2006; Ohashi *et al.*, 2005; European references - Cinco *et al.*, 1997; Guy *et al.*, 1998; Ogden *et al.*, 1998; Fingerle *et al.*, 1999; Leutenegger *et al.*, 1999; Petrovec *et al.*, 1999; Pusterla *et al.*, 1999a; Alekseev *et al.*, 2001b; Christova *et al.*, 2001; Jenkins *et al.*, 2001; Liz *et al.*, 2001; Oteo *et al.*, 2001; Grzeszczuk *et al.*, 2002, 2004b; Oehme *et al.*, 2002; Morozova *et al.*, 2002; Santino *et al.*, 2002; Spitalska *et al.*, 2002; Derdakova *et al.*, 2003; Hildebrandt *et al.*, 2003; Makinen *et al.*, 2003; Sixl *et al.*, 2003; Skotarczak *et al.*, 2003; Hartelt *et al.*, 2004; Polin *et al.*, 2004; Stanczak *et al.*, 2004; Tomasiewicz *et al.*, 2004; Cisak *et al.*, 2005; De la Fuente *et al.*, 2005a; Rar *et al.*, 2005; Ferquel *et al.*, 2006; Grzeszczuk & Stanczak, 2006; Halos *et al.*, 2006; Mantelli *et al.*, 2006; Piccolin *et al.*, 2006; Shpynov *et al.*, 2006;
North American references - Magnarelli *et al.*, 1995b; Pancholi *et al.*, 1995; Barlough *et al.*, 1997a; Chang *et al.*, 1998; Daniels *et al.*, 1998; Varde *et al.*, 1998; Kramer *et al.*, 1999; Lane *et al.*, 2001, 2004; Fang *et al.*, 2002; Massung *et al.*, 2002, 2003a; Courtney *et al.*, 2003; Holden *et al.*, 2003; Adelson *et al.*, 2004; Dolan *et al.*, 2004; Caporale *et al.*, 2005; Fritz *et al.*, 2005; Schulze *et al.*, 2005;
**Total number of tested ticks.

The patchy distribution of *A. phagocytophilum* in nature is multifactorial and may depend on the existence of both competent vector ticks and reservoir hosts. In many regions *Ixodes* ticks are found beyond the areas of pathogen endemicity. The discrepancies between vector ticks and pathogen distribution are not well understood but might be related to feeding behaviour and reservoir-host dynamics (Swanson *et al.*, 2006). In fact, the apparent lack of *A. phagocytophilum* transovarial transmission in ticks directs attention to competent vertebrates, especially those which represent a feeding support for larvae and nymphs, as playing a critical role in the maintenance of agent's active cycles in nature.

Members of *I. persulcatus/ricinus* complex are usually characterized by a permissive feeding behaviour with a broad range of potential vertebrate hosts, including avian, reptilian, and numerous mammalian species (Sonenshine, 1991). Immature stages often parasitize rodents and other small animals, contrasting with adult ticks that quest medium to large-size mammals. The role of reptiles in *A. phagocytophilum* cycle is largely unknown. Several researchers support the idea that although birds seem to lack systemic infections, they promote agent exchange between cofeeding ticks and contribute to the dispersion of infecting ticks (Alekseev *et al.*, 2001a; Bjoersdorff *et al.*, 2001; Daniels *et al.*, 2002). However, a recent study has detected *A. phagocytophilum* in several bird blood specimens in Spain (De la Fuente *et al.*, 2005a). But the most relevant role is most certainly played by mammals, especially rodents and other small mammals that are often parasitized by immature stages of ticks. Several species of rodents have been already implicated in *A. phagocytophilum* cycle as primary reservoirs, especially *Peromyscus leucopus* in the Northeastern and North Central US (Telford *et al.*, 1996; Levin *et al.*, 2002), *Neotoma mexicana*, *N. fuscipes*, and *Peromyscus maniculatus* in Western US (Zeidner *et al.*, 2000; Burkot *et al.*, 2001; Foley *et al.*, 2002), *Myodes* (formerly *Clethrionomys*) *glareolus*, *Apodemus flavicollis* and *A. sylvaticus* in Europe (Odgen *et al.*, 1998; Liz *et al.*, 2000; Bown *et al.*, 2003), and *A. agrarius* in Asia (Chae *et al.*, 2003). Natural infections have also been reported from a wide range of other mammalians that may as well integrate the primary transmission cycle of *A. phagocytophilum* or even settle parallel cycles that would help to perpetuate the agent in nature (Table 4 and Figure 7). Examples of parallel transmission cycles have been observed between lagomorphs and rodents and their host-specific ticks, such as *Sylvilagus floridanus* and *I. dentatus* in Nantucket Island, Massachusetts (Goethert & Telford, 2003), *Neotoma sp*. and *I. spinipalpis* in Colorado (Zeidner *et al.*, 2000; Burkot *et al.*, 2001) and *Myodes glareolus*, *Microtus agrestis* and *I. trianguliceps* in United Kingdom (Bown *et al.*, 2003, 2006).

### 1.2.5. OTHER *IXODES*-BORNE AGENTS

*A. phagocytophilum* geographic distribution may overlap those of other pathogenic agents either because they share the same reservoir hosts and/or the vectors ticks. This latter aspect is of particular interest regarding the epidemiology of concurrent tick-borne diseases. Besides *A. phagocytophilum* transmission, members of *I. persulcatus/ricinus* complex are also potential or

confirmed vectors of several other agents with Public Health relevance, including *Anaplasma* spp., *Babesia* spp., *Bartonella* spp., *B. burgdorferi s.l.*, *Coxiella burnetii*, *Francisella tularensis*, *Rickettsia helvetica*, *Toxoplasma gondii* and some tick-borne viruses (Table 5) (Rehacek *et al.*, 1994; Estrada-Pena & Jongejan *et al.*, 1999; Parola & Raoult, 2001b; Gray *et al*., 2002; Sonogo *et al.*, 2003; Sroka *et al.*, 2003; Holden *et al.*, 2006). Moreover, newly described agents with unknown pathogenicity have also been associated to these tick species, such as candidatus *Neoehrlichia mikurensis* ("*Ehrlichia walkerii*"), *Rickettsia monacensis* and IRS3 (Márquez *et al.*, 1998; Sekeyova *et al.*, 2000; Simser *et al.*, 2002; Brouqui *et al.*, 2003; Kawahara *et al.*, 2004).

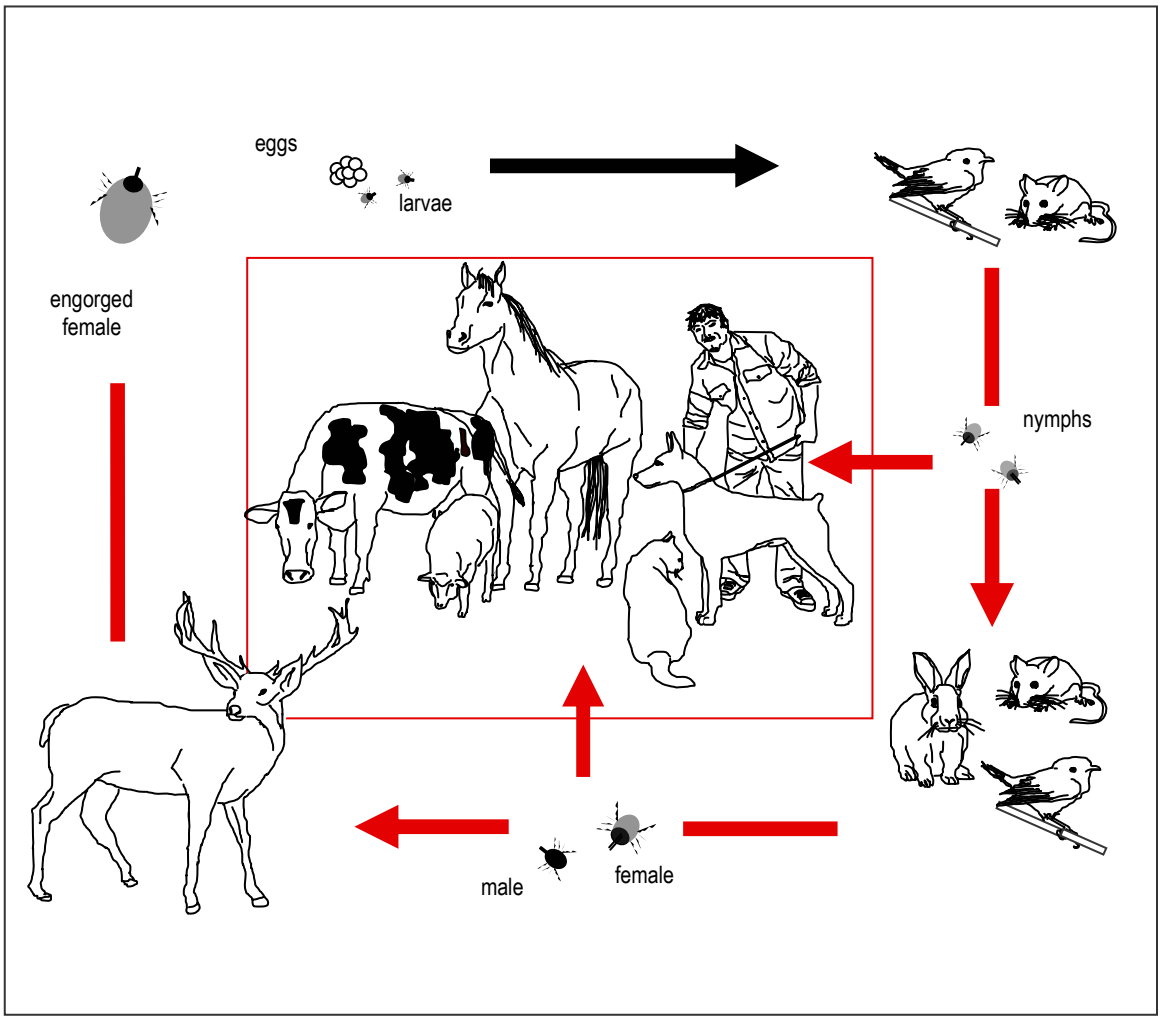



FIGURE 7. Schematic representation of *A. phagocytophilum* transmission cycle.

potentially infected ticks; potentially infective bite; non-infected ticks; non-infective bite.

TABLE 4. *A. phagocytophilum* infection in wild mammals detected by PCR-based studies.

| Region/Order | Vertebrate species (common name) | References |
|---|---|---|
| Asia | | |
| Artiodactyla | *Cervus nippon nippon* (wild sika deer)<br>*C. nippon yesoensis* (wild sika deer) | Kawahara *et al.*, 2006 |
| Rodentia | *Apodemus agrarius* (black-striped field mouse)<br>*A. peninsulae* (korean field mouse)<br>*Tamias sibiricus* (siberian chipmunk) | Chae *et al.*, 2003; Cao *et al.*, 2006; Kim *et al.*, 2006 |
| Soricomorpha | *Crocidura lasiura* (ussuri white-toothed shrew) | Kim *et al.*, 2006 |
| Europa | | |
| Artiodactyla | *Capreolus capreolus* (roe deer)<br>*Cervus elaphus* (red elk)<br>*Rupicapra rupicapra* (chamois)<br>*Sus scrofa* (wild boar) | Alberti *et al.*, 2000; Stuen *et al.*, 2001; Liz *et al.*, 2002; Petrovec *et al.*, 2002, 2003; Oporto *et al.*, 2003; Polin *et al.*, 2004; Skarphedinsson *et al.*, 2005 |
| Rodentia | *Apodemus agrarius* (black-striped field mouse)<br>*A. flavicollis* (yellow-necked mouse)<br>*A. sylvaticus* (long-tailed field mouse)<br>*Microtus agrestis* (field vole)<br>*M. oeconomus* (tundra vole)<br>*Myodes glareolus** (bank vole)<br>*Rattus rattus* (black rat) | Ogden *et al.*, 1998; Liz *et al.*, 2000; Bown *et al.*, 2003, 2006; Christova & Gladnishka, 2005; Grzeszczuk *et al.*, 2006a |
| Soricomorpha | *Sorex araneus* (eurasian shrew) | Liz *et al.*, 2000 |
| North America | | |
| Artiodactyla | *Cervus elaphus nannodes* (tule elk)<br>*Odocoileus hemionus columbianus* (black-tailed deer)<br>*O. hemionus hemionus* (mule deer)<br>*O. virginianus* (white-tailed deer) | Belongia *et al.*, 1997; Foley *et al.*, 1998; Massung *et al.*, 2005a; Michalski *et al.*, 2006 |
| Carnivora | *Canis latrans* (coyote)<br>*Procylon lotor* (Raccoon)<br>*Puma concolor* (mountain lion)<br>*Ursus americanus* (american black bear) | Foley *et al.*, 1999b; Drazenovich *et al.*, 2006; Pusterla *et al.*, 2000; Levin *et al.*, 2002 |
| Lagomorpha | *Lepus californicus* (black-tailed jackrabbit)<br>*Sylvilagus audubonii* (desert cottontails rabbit)<br>*S. floridanus* (eastern cottontails rabbit) | Goethert & Telford, 2003; Yabsley *et al.*, 2006 |
| Rodentia | *Microtus ochrogaster* (prairie vole)<br>*Myodes gapperi*** (southern red-backed vole)<br>*Neotoma fucipes* (dusky-footed wood rat)<br>*N. mexicana* (mexican wood rat)<br>*Peromyscus leucopus* (white-footed mouse)<br>*P. maniculatus* (deer mouse)<br>*P. truei* (pinyon mouse)<br>*Reithrodontomys megalotis* (western harvest mouse)<br>*Sciurus griseus (*western grey squirrel)<br>*Spermophilus lateralis* (golden-mantled ground squirrel)<br>*Tamias minimus* (least chipmunk)<br>*T. striatus* (eastern chipmunk) | Walls *et al.*, 1997; Yeh *et al*., 1997; Foley *et al.*, 1999b; Nicholson *et al.*, 1999; Stafford *et al.*, 1999; Zeidner *et al.*, 2000; Burkot *et al.*, 2001; Castro *et al.*, 2001; DeNatale *et al.*, 2002; Levin *et al.*, 2002; Massung *et al.*, 2002; Lane *et al.*, 2005; Drazenovich *et al.*, 2006; Nieto *et al*., 2007 |

* Formerly *Clethrionomys glareolus;* ** formerly *Clethrionomys gapperi*.

TABLE 5. Members of *Ixodes persulcatus/ricinus* complex as potential (*) and confirmed vectors for other tick-borne agents.

| Pathogen | Disease | Affected hosts | Vectors |
|---|---|---|---|
| Virus | | | |
| CCHF virus | Crimean–Congo hemorrhagic fever (CCHF) | Humans | **I. ricinus* |
| Looping ill virus | Looping ill | Sheep | *I. ricinus* |
| TBE virus | Tick-borne encephalitis (TBE) | Humans | *I. ricinus*, *I. persulcatus* |
| Bacteria | | | |
| *Anaplasma bovis* | Anaplasmosis | Bovines | **I. ricinus* |
| *A. marginale* | Anaplasmosis | Bovines | **I. ricinus* |
| *Bartonella henselae* | Bartonellosis | Humans/ animals | **I. ricinus*; **I. pacificus*, **I. scapularis* |
| *Borrelia burgdorferi s.l.* | Lyme borreliosis (LB) | Humans/ animals | |
| *Borrelia afzelii* | | | *I. ricinus, I. persulcatus* |
| *B. burgdorferi s.s.* | | | All four species |
| *B. garinii* | | | *I. ricinus, I. persulcatus* |
| *B. lusitaniae* | | | *I. ricinus* |
| *B. valaisiana* | | | *I. ricinus* |
| *Coxiella burnetii* | Q fever | Humans/ animals | **I. ricinus* |
| *Ehrlichia walkerii* | Unknown | Unknown | *I. ricinus* |
| *Francisella tularensis* | Tularemia | Humans/ animals | **I. ricinus* |
| *Rickettsia helvetica* | Febrile illness | Humans | *I. ricinus* |
| *R. monacensis*/ IRS3 | Unknown | Unknown | **I. ricinus* |
| Protozoa | | | |
| *Babesia divergens* | Babesiosis | Humans/ bovines | *I. ricinus* |
| *B. microti* | Babesiosis | Humans | *I. scapularis* |
| *B. odocoilei* | Babesiosis | Wild ruminants | **I. ricinus, I. scapularis* |
| *Toxoplasma gondii* | Toxoplasmosis | Humans/ animals | **I. ricinus* |

Several studies have attempted to identify simultaneous infection in *I. persulcatus/ricinus* complex but available data are essentially the result of focused detection of *B. burgdorferi s.l.* and *Babesia* spp. and/or *A. phagocytophilum* (www.pubmed.gov). Thus, the true prevalence of other coinfecting pathogens among *Ixodes* ticks remains largely unknown in the majority of geographic locations. These molecular studies have evidenced that although single infections are substantially more common, the possibility for coinfections exists. In a recent revision of published articles about this subject, Swanson and coworkers (2006) have shown that dual infection with any combination of *B. burgdorferi s.l.*, *B. microti*, and *A. phagocytophilum* occurs in 1% to 28% of *Ixodes* ticks from regions of LB endemicity in the US and in <1% to 13% of sampled European *Ixodes* ticks. Triple infection is rarely detected in geographic regions where all these tick-borne disases are endemic and likely represents an incident occurrence of <1%. Moreover, *A. phagocytophilum* coinfection

with *Babesia odocoilei*, *Bartonella henselae*, *Bartonella* spp., and spotted fever rickettsiae have also been sporadically reported in ticks (Spitalska *et al.*, 2002; Holden *et al.*, 2003,. 2006; Adelson *et al.*,2004; Hartelt *et al.*, 2004; Halos *et al.*, 2006; Steiner *et al.*, 2006). Table 6 summarizes the prevalences of *A. phagocytophilum* coinfections detected in ticks.

### 1.2.6. VARIANT STRAINS AND DISEASE

*A. phagocytophilum* presents a minor degree of variation in the nucleotide sequences of conserved genes, such as *rrs*, *groESL*, and *ank*A[2] which has strengthened its unification into a single species (Sumner *et al.*, 1997; Caturegli *et al.*, 2000; Massung *et al.*, 2000; Dumler *et al.*, 2001; Yu *et al.*, 2001). Nevertheless, the existence of sequence polymorphisms has been a matter of interest in the scientific community suggesting the occurrence of variant strains with distinct geographic origin, reservoir hosts and pathogenicity (Shukla *et al.*, 2007).

Phylogenetic analysis of the *ankA* gene sequences has separated *A. phagocytophilum* into three distinct clades, representing the US Northeasthern and Upper Midwesthern strains, and European strains (Massung *et al.*, 2000). But more genetic heterogeneity may exist, as demonstrated by Von Loewenich and coworkers (2003a) with the description of unexpected *ankA* diversity in infected German ticks. The analysis of *groESL* has reinforced the association of variability with geographic origin but it also suggested the existence of distinct *A. phagocytophilum* lineages related with specific reservoir hosts, possibly the result of coevolution (Sumner *et al.*, 1997; Petrovec *et al.*, 1999; Chae *et al.*, 2000; Bjoersdorff *et al.*, 2002a; Petrovec *et al.*, 2002; Von Loewenich *et al.*, 2003a; Polin *et al.*, 2004). Moreover the analysis of the most conservative *rrs*, has shown that only two genotypes of *A. phagocytophilum* have thus far been associated with human disease: the prototype described by Chen and coworkers (1994), that occur in both Europe and North America (Northeast and Upper Midwest), and the strain described by Foley and coworkers (1999a) in California. These strains, referred to as Ap-ha (GenBank acession nos U02521; AF093788/ AF093789), are also pathogenic for horses, dogs and possible for cats, but less commonly associated with ruminants. The dominant genotype responsible for ruminant disease is referred to as Ap-variant 1 (GenBank acession no M73220) (Bjoersdorff *et al.*, 2002a;

[2] Gene encoding AnkA, the 153-160 kDa protein with several tandemly repeated ankyrin motifs, as previously mentioned.

TABLE 6. Prevalence of *A. phagocytophilum* coinfections in questing *Ixodes* ticks detected by PCR-based methods.

| Region | Reference | *Ixodes* species | No ticks sampled | Coinfecting agents* | Prevalence (%) of infection | Prevalence (%) of coinfection** |
|---|---|---|---|---|---|---|
| Asia | | | | | | |
| China | Cao *et al.*, 2003 | *I. persulcatus* | 1,345 | Ap/Bb | Ap 4.6; Bb 33.8 | Ap/Bb 0.5 |
| Europe | | | | | | |
| Bulgaria | Christova *et al.*, 2001 | *I. ricinus* | 112 | Ap/Bb | Ap 33.9; Bb 32.1 | Ap/Bb 13.4 |
| Germany | Fingerle *et al.*, 1999 | *I. ricinus* | 492 | Ap/Bb | Ap 1.6; Bb 36.2 | Ap/Bb 0.8 |
| | Oehme *et al.*, 2002 | *I. ricinus* | 898 | Ap/Bb | Ap 2.9; Bb 20.4 | Ap/Bb 0.8 |
| | Hildebrandt *et al.*, 2003 | *I. ricinus* | 305 | Ap/Bb | Ap 2.3; Bb 11.1 | Ap/Bb 0.7 |
| Italy | Cinco *et al.*, 1997 | *I. ricinus* | 86 | Ap/Bb | Ap 24.4; Bb 19.8 | Ap/Bb 8.14 |
| Poland | Skotarczak *et al.*, 2003 | *I. ricinus* | 514 | Ap/Bb; Ap/Bm; Ap/Bb/Bm | Ap 4.7; Bb 15.8; Bm 13.0 | Ap/Bb 1.4; Ap/Bm 0.8; Ap/Bb/Bm 0.6 |
| | Stanczak *et al.*, 2004 | *I. ricinus* | 303 | Ap/Bb; Ap/Bm | Ap 19.5; Bb 29.7; Bm 3.6 | Ap/Bb 8.3; Ap/Bm 2.0 |
| | Stanczak *et al.*, 2002 | *I. ricinus* | 424 | Ap/Bb | Ap 19.2; Bb 11.6 | Ap/Bb 5.0 |
| Russia | Morozova *et al.*, 2002 | *I. persulcatus* | 150 | Ap/Bb | Ap 8.0; Bb 38.0 | Ap/Bb 6.0 |
| Slovakia | Spitalska *et al.*, 2002 | *I. ricinus* | 137 | Ap/RSFG | Ap 4.4; SFGR 10.9 | Ap/SFGR 0.7 |
| | Derdakova *et al.*, 2003 | *I. ricinus* | 60 | Ap/Bb | Ap 8.3; Bb 38.3 | Ap/Bb 5.0 |
| Switzerland | Leutenegger *et al.*, 1999 | *I. ricinus* | 100 | Ap/Bb | Ap 2.0; Bb 49.0 | Ap/Bb 2.0 |
| United Kingdom | Guy *et al.*, 1998 | *I. ricinus* | 60 | Ap/Bb | Ap 7.0; Bb 37.0 | Ap/Bb 1.7 |
| North America | | | | | | |
| California | Holden *et al.*, 2003 | *I. pacificus* | 776 | Ap/Bb | Ap 6.2; Bb 5.7 | Ap/Bb 1.0 |
| | Lane *et al.*, 2004 | *I. pacificus* | 158 | Ap/Bb | Ap 3.2; Bb 3.8 | Ap/Bb 1.3 |
| | Holden *et al.*, 2006 | *I. pacificus* | 168 | Ap/Bh-like | Ap 28.6; Bh-like 6.5 | Ap/Bh-like 3.0 |
| New Jersey | Varde *et al.*, 1998 | *I. scapularis* | 100 | Ap/Bb; Ap/Bm | Ap 17.0; Bb 43.0; Bm 5.0 | Ap/Bb 6.0; Ap/Bm 2.0 |
| | Adelson *et al.*, 2004 | *I. scapularis* | 107 | Ap/Ba spp; Ap/Bb/Ba; Ap/Bm/Ba; | Ap 1.9; Bb 33.6; Ba 34.5; Bm 8.4 | Ap/Ba 0.9; Ap/Bb/Ba 0.9; Ap/Bm/Ba 0.9 |
| | Schulze *et al.*, 2005 | *I. scapularis* | 147 | Ap/Bb | Ap 6.1; Bb 50.3 | Ap/Bb 2.7 |
| New York | Chang *et al.*, 1998 | *I. scapularis* | 229 | Ap/Bb | Ap 9.0; Bb 54.0 | Ap/Bb 4.0 |
| | Daniels *et al.*, 1998 | *I. scapularis* | 1,268 | Ap/Bb | Ap 13.5; Bb 15.1 | Ap/Bb 2.2 |
| | Schauber *et al.*, 1998 | *I. scapularis* | 188 | Ap/Bb | Ap 42.6; Bb 66.0 | Ap/Bb 28.2 |
| Pennsylvania | Courtney *et al.*, 2003 | *I. scapularis* | 454 | Ap/Bb | Ap 17.8; Bb 41.2 | Ap/Bb 3.5 |
| Wisconsin | Pancholi *et al.*, 1995 | *I. scapularis* | 89 | Ap/Bb | Ap 7.9; Bb 11.2 | Ap/Bb 2.2 |

* Ap *Anaplasma phagocytophilum*; Ba *Bartonella* spp.; Bb *Borrelia burgdorferi s.l.*; Bm *Babesia microti*; Bh-like *Bartonella henselae*–like; SFGR spotted fever group *Rickettsia* spp.;
** Coinfection data overlaps with the single-pathogen prevalence percentages.

Stuen *et al.*, 2002b, 2003a, 2003b, 2005; Von Loewenich *et al.*, 2003b; Poitout *et al.*, 2005; Massung *et al.*, 2006). Both Ap-ha and Ap-variant 1 have been detected in vector ticks and hosts but the existence of distinct enzootic cycles is suggested by the association of Ap-ha to small mammals and Ap-variant 1 to wild ruminants (Belongia *et al.*, 1997; Massung *et al.*, 1998, 2002, 2003a, 2003b, 2005a, 2006; Leutenegger *et al.*, 1999; Zeidner *et al.*, 2000; Petrovec *et al.*, 2002; Cao *et al.*, 2003; Courtney *et al.*, 2003; Sreter *et al.*, 2004; Tate *et al.*, 2005; Michalski *et al.*, 2006). However, the detection of coinfection in questing ticks indicates that those cycles are linked to some extent and both strains are capable of coexisting in the same tick vector (Massung *et al.*, 2002). Several other genetic variants of *A. phagocytophilum* have been described, some of those with unknown pathogenic potential (Walls *et al.*, 1997; Baumgarten *et al.*, 1999; Schouls *et al.*, 1999; Cao *et al.*, 2000, 2003; Massung *et al.*, 2002; Michalski *et al.*, 2006).

The distinction between ruminant and non-ruminant lineages of *A. phagocytophilum* was initially suggested by experimental cross-infections [(Barlough *et al.*, 1995; Madigan *et al.*, 1995; Stuen *et al.*, 1998 *in* Stuen *et al.*, 2002b); Pusterla *et al.*, 1999b, 2001]. A thorough understanding of the significance of *A. phagocytophilum* genetic variants is still far from complete, but their impact on both human and animal disease is already a matter of debate. Based on a 4-year field work study Massung and coworkers (2002) have hypothesized that variants strains of *A. phagocytophilum* may interfere with the maintenance and transmission of those causing human disease, resulting in a lower incidence of HGA in regions where different variants are common. Moreover, Stuen and coworkers (2005) have recently shown direct interference of *A. phagocytophilum* strains by the dominance of the genotype Ap-variant 1 over Ap-variant 2 in experimentally infected sheep. As discussed by those researchers, the reason for this dominance is unknown, but factors such as growth rate, immunogenicity, receptor competition, and antigenic variation may be involved in the infectivity and interaction of *A. phagocytophilum* variants. Further research is needed to investigate the role of competitive interactions between genetic variants of *A. phagocytophilum* that may modulate human and animal disease in each geographic area.

### 1.2.6.1. TICK-BORNE FEVER

(*A. phagocytophilum* synonyms: *Rickettsia phagocytophila*, *Cytoecetes phagocytophila*, *Ehrlichia phagocytophila, Anaplasma phagocytophila*)

Tick-borne fever (TBF) or pasture fever was first recognized in 1932 as a disease of sheep grazed on tick-infested pastures in Great Britain (Gordon *et al.*, 1932 *in* Dumler *et al.*, 2001). In the preceding years considerable information was acquired concerning the host range, pathogenicity and vector relationships. TBF affects domestic ruminants, chiefly sheep and cattle, in several European countries but is rarely reported in North America. Only a sporadic case of the disease was described in a llama (Barlough *et al.*, 1997b) which suggests that the North American Ap-Variant 1 or other ruminant strain have evolved into a less pathogenic form than the European counterparts. TBF is characterized by fever, apathy, anorexia, tachypnea, cough, nasal discharge, and reluctance to move [(Gordon *et al.*, 1932; Hudson, 1950; Foggie, 1951) *in* Dumler *et al.*, 2001]. Additionally, abortion commonly occurs if naïve pregnant sheep are introduced into pastures infested with vector ticks (Jones & Davis, 1995 *in* Borjesson & Barthold, 2002; Garcia-Perez *et al.*, 2003). Indirect losses are also associated with a decrease in milk production and reduced growth rate in both calves and lambs [(Gordon *et al.*, 1932; Hudson, 1950; Foggie, 1951) *in* Dumler *et al.*, 2001; Stuen *et al.*, 2002a]. The disease is usually moderate, but serious complications that may lead to death have also been observed. The most serious problem associated with TBF, especially in sheep, is the increase in severity of concurrent louping ill and the predisposition to secondary infections, such as staphylococcal abscesses, septicemia, and pasteurellosis, among others [(Gordon *et al.*, 1932; Foggie, 1951, 1956; Gronstol & Ulvund, 1977; Gilmour *et al.*, 1982; Batungbacal & Scott, 1982) *in* Borjesson & Barthold, 2002; Stuen *et al.*, 2003b].

### 1.2.6.2. EQUINE GRANULOCYTIC ANAPLASMOSIS

(*A. phagocytophilum* synonym: *Ehrlichia equi, Anaplasma phagocytophila*)

Equine granulocytic (formerly ehrlichiosis) anaplasmosis (EGA) was initially reported in 1969 associated to severe disease in horses in the foothills of the Sacramento Valley, California [(Gribble, 1969; Stannard *et al.*, 1969) *in* Dumler *et al.*, 2001]. Since then, the disease has been observed chiefly in California but sporadic cases are also reported in other parts of US and Europe (Engvall *et al.*, 1996; Madigan *et al.*, 1996; Pusterla *et al.*, 1998a; Bullok *et al.*, 2000;

Von Loewenich *et al.*, 2003b; Alberti *et al.*, 2005). EGA is usually a mild.to moderately severe disease characterized by fever, depression, anorexia, icterus, limb oedema, petechiation, ataxia and reluctance to move.

#### 1.2.6.3. OTHER GRANULOCYTIC ANAPLASMOSIS

The susceptibility of dogs and cats to *A. phagocytophilum* was first demonstrated in an experimental study (Lewis *et al.*, 1975 *in* Greig *et al.*, 1996) and later confirmed to occur naturally. In dogs, the disease was originally documented in California after the successful transmission of the agent from febrile, thrombocytopenic dogs to horses by blood transfusion (Madewell & Gribble 1982 *in* Greig *et al.*, 1996). Natural infection in domestic felines was initially recognized in Sweden (Bjoersdorff *et al.*, 1999c). In both cases, clinical disease was also found in other parts of North America and Europe (Engvall *et al.*, 1996; Greig *et al.*, 1996; Tozon *et al.*, 2003; Lappin *et al.*, 2004; Alberti *et al.*, 2005; Lester *et al.*, 2005; Shaw *et al.*, 2005). In general, the disease ranges from subclinical to mild febrile illness accompanied by depression, lethargy, and classic hematologic alterations.

Regarding human infection by *A. phagocytophilum* more detailed consideration is given below.

## 1.3. HUMAN GRANULOCYTIC ANAPLASMOSIS

### 1.3.1. DEFINITION

*A. phagocytophilum* infection in humans, previously known as human granulocytic or granulocytotropic ehrlichiosis (HGE), is now called human granulocytotropic anaplasmosis (HGA). The synonym, HGE agent, was originally used to designate the etiology of the disease.

### 1.3.2. EPIDEMIOLOGY

Epidemiologic information regarding HGA is still limited. The available data seem to reflect the geographic distribution of *A. phagocytophilum* associated with human disease, competent tick vectors and reservoir hosts, their seasonal activity, and also human behavior that places persons at risk of tick attachment, and individual factors that determine the subsequent development of the disease. Understanding disease trends may help target prevention efforts and assist physicians in accurate and timely HGA diagnosis.

To date, HGA descriptions are restricted to the Northern hemisphere. As previously stated, the majority of patients have been diagnosed in the US, although a limited number of cases have also been reported from some European countries. In the US, HGA was originally described in 1994 from a series of patients residing in Wisconsin and Minnesota (Bakken *et al.*, 1994). In the following years, HGA was increasingly recognized also in other states, such as Connecticut, New York, Massachusetts and Rhode Island (Hardalo *et al.*, 1995; Telford *et al.*, 1995; CDC, 1995; Aguero-Rosenfeld *et al.*, 1996; Bakken *et al.*, 1996c; Goodman *et al.*, 1996; McQuiston *et al.*, 1999; Belongia *et al.*, 2001a; Gardner *et al.*, 2003). The disease became nationally notifiable in 1999 and is now in the rank of the 30$^{th}$ most important infectious diseases regarding the total number of incident cases reported to the Centers for Disease Control and Prevention (CDC, 2006). HGA cases are reported by State Health Departments to CDC through both the National Electronic Telecommunications System for Surveillance (NETSS) and Tick-Borne Rickettsial Disease Case Report Form (CRF). According to NETSS (CDC, 2001-2006), more than 2.000 cases have been reported in the six years of national surveillance representing an average annual incidence of 0.15

per $10^5$ inhabitants (Figure 8). The disease occurs mainly in the Upper Midwest, and parts of New England and the Mid-Atlantic States, which is consistent with previous surveillance reports (CDC, 1998; McQuiston *et al.*, 1999). In those areas, HGA is even more prevalent than other tick-borne diseases, such as Rocky Mountain spotted fever (RMSF)[4] and HME, and is the second most reported tick bite-associated illness after LB (CDC, 2001-2006). The total number of HGA cases reported per state during 1999-2004 is presented in figure 9. Because nationally reportable diseases are recorded by state of residence rather than state of exposure, undoubtedly some cases might be incorrectly classified. Underreporting and inadequate diagnosis of HGA are also referred to as important limitations of the current passive surveillance system which might account for an incomplete overall distribution and regional prevalence of the disease (IJdo *et al.*, 2000; Demma *et al.*, 2005).

The first European case of HGA was described in 1997 in Slovenia (Petrovec *et al.*, 1997), and more than 50 patients have been identified since then. The majority of cases have occurred in Central and Northern Europe, in areas at high prevalence of tick-borne encephalitis (TBE)[5] and LB. Slovenia and Sweden account for the highest record of the disease (Bjoersdorff *et al.*, 1999a, 2002b; Laferl *et al.*, 1999; Arnez *et al.*, 2001; Karlsson *et al.*, 2001; Lotric-Furlan *et al.*, 2006) although sporadic cases have also been reported in Austria (Walder *et al.*, 2003a, 2006a), Czech Republic (Hulinska *et al.*, 2002), Croatia (Misić-Majerus *et al.*, 2006), Estonia (Prukk *et al.*, 2003), France (Remy *et al.*, 2003), Italy (Ruscio & Cinco, 2003; De la Fuente *et al.*, 2005b; Beltrame *et al.*, 2006; Mastrandrea *et al.*, 2006), Netherlands (Van Dobbenburgh *et al.*, 1999), Norway (Bjoersdorff *et al.*, 1999a, Kristiansen *et al.*, 2001), Poland (Tylewska-Wierzbanowska *et al.*, 2001; Grzeszczuk *et al.*, 2006b), and Spain (Oteo *et al.*, 2000; Garcia *et al.*, 2006). The geographic distributions of European HGA cases that have been reported since 1997 are presented in figure 10.

Seroepidemiological studies, however, suggest that human infection by *A. phagocytophilum* might be more common than clinical illness. The presence of antibodies against *A. phagocytophilum* has been reported from endemic areas in US and Europe and also from other European and Asiatic countries where HGA is not known to occur (Park *et al.*, 2003b; Ongut *et al.*, 2006; Walder *et al.*, 2006b). Reported seroprevalence rates range from zero or very low to up to 25% of the examined populations (Table 7). Generally, the proportion of seropositive persons increases with age and is higher for at-risk populations, such as forestry workers, persons with a

[4] Rocky Mountain spotted fever (RMSF) is caused by the α-proteobacteria *Rickettsia rickettsii*.
[5] Tick-borne encephalitis (TBE) is caused by the infection of a Flavivirus that received the same designation as the disease (TBE vírus).

history of tick bite(s) and/or patients with LB and those with TBE, than in control subjects who are not (or who are less) exposed to ticks (Magnarelli *et al.*, 1995a; Bakken *et al.*, 1998b; Nuti *et al.*, 1998; Pusterla *et al.*, 1998b; Wittesjo *et al.*, 2001; Aguero-Rosenfeld *et al.*, 2002; Grzeszczuk *et al.*, 2004b).

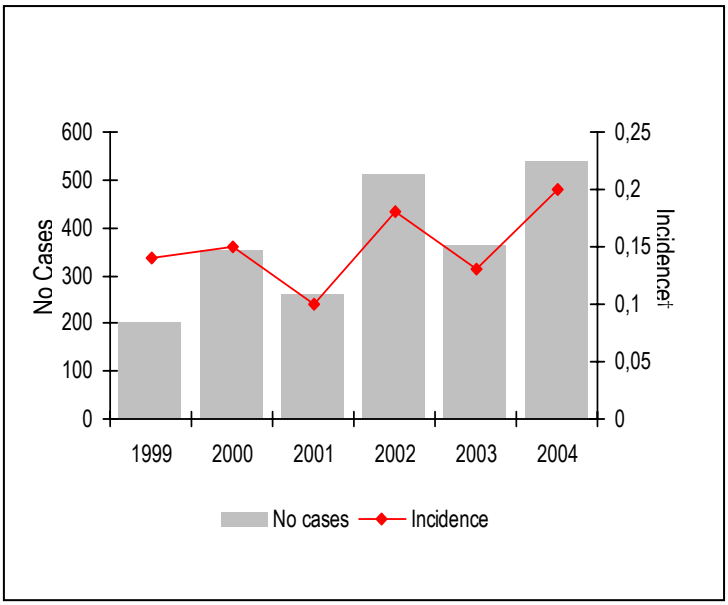
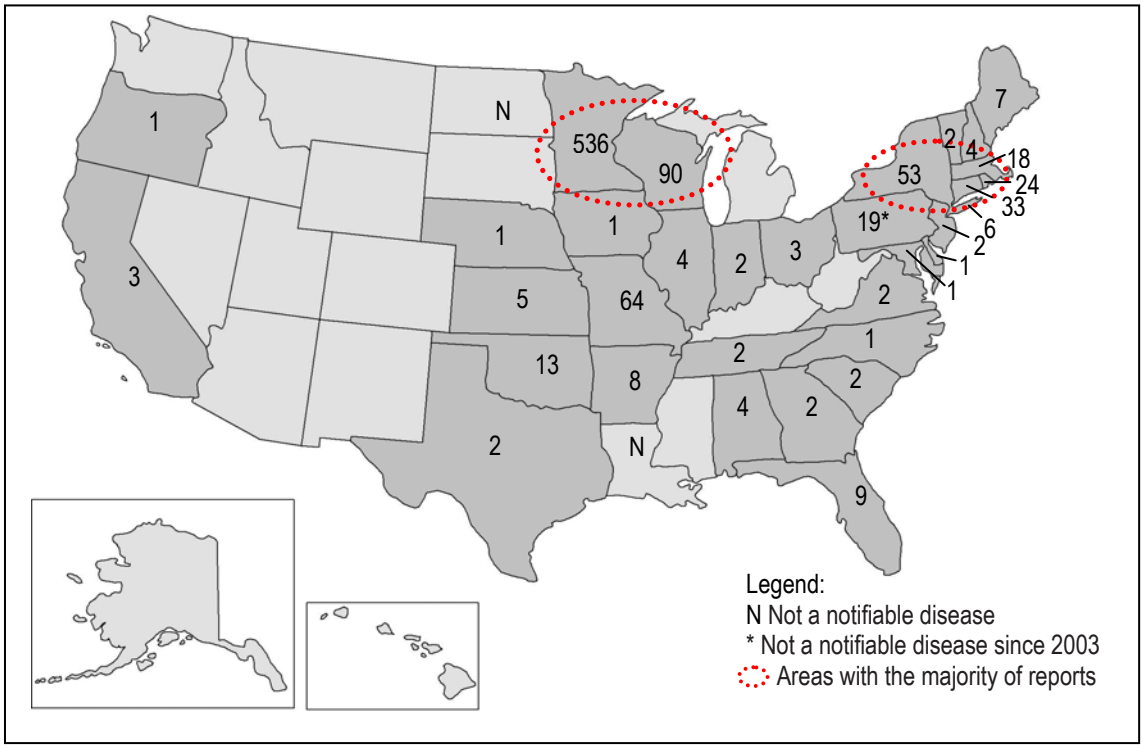



FIGURE 8. Number of HGA cases reported to NETSS, US 1999-2004 (adapted from CDC 2001-2006).
Available from: http://www.cdc.gov/mmwr/summary.html

†Note. Incidence is presented per 100,000 inhabitants, based on data for the US total resident population. Population estimates are from the Bureau of the Census.
Available from: http://www.census.gov/

FIGURE 9. Total number of HGA cases reported to NETSS by State, US 1999-2004 (adapted from CDC 2001-2006).
Available from: http://www.cdc.gov/mmwr/summary.html



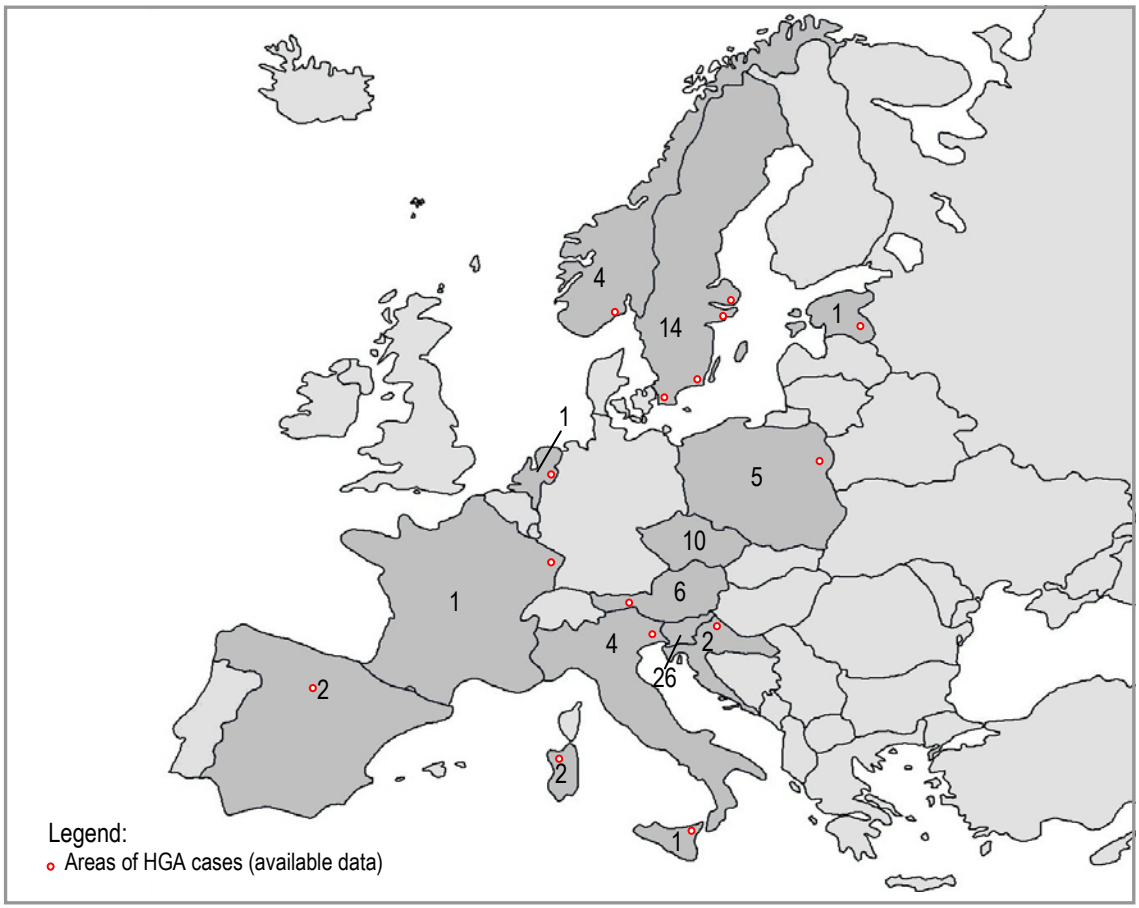



FIGURE 10. Total number of HGA cases reported in each European country since 1997 (based in a medline search http://www.pubmed.gov).

It is believed that asymptomatic or subclinical infections may contribute to the broad seropositivity. In four prospective serological studies up to 11% seroconversions were detected in individuals with a high degree of exposure without evidence of clinical illness, over a single tick season (Hilton *et al.*, 1999; Wittesjo *et al.*, 2001; Woessner *et al.*, 2001; Grzeszczuk *et al.*, 2004a). Nevertheless, it still remains unclear whether the discrepancy between the seroprevalence and incidence rate results from underdiagnosis of mild infections, asymptomatic serologic responses to *A. phagocytophilum* variants not associated with HGA, or even infections with other agents that produce cross-reactive serologic responses.

Exposure to tick bites is considered the most common route of human infection by *A. phagocytophilum*, although sporadic cases of HGA have been reported after perinatal transmission or contact with infected animal blood (Bakken *et al.*, 1996a; Horowitz *et al.*, 1998c). Transfusion transmission is also biologically plausible, but only a single possible case of transfusion-related HGA has been identified (McQuiston *et al.*, 2000; Kalantarpour *et al.*, 2000; Leiby & Gill, 2004).

TABLE 7. Meta-analysis of *A. phagocytophilum* seroprevalence (%) among various human populations based in seropositivity obtained from immunofluorescence assay* (adapted from Dumler, 2005).

| | Cumulative Analysis Mean (Number of Subjects) | Median Analysis | | | Number Studies |
|---|---|---|---|---|---|
| | | Median | Maximum | Minimum | |
| Overall | | | | | |
| Asia[a] | 5.9 (816) | 3.7 | 11.1 | 2.3 | 2 |
| Europe[b] | 7.0 (8,956) | 7.3 | 23.0 | 0.0 | 26 |
| North America[c] | 9.7 (4,452) | 8.6 | 35.6 | 0.0 | 14 |
| At-risk[d] | | | | | |
| Asia | 11.1 (271) | 11.1 | 11.1 | 11.1 | 1 |
| Europe | 9.3 (5,517) | 8.8 | 23.0 | 0.0 | 22 |
| North America | 10.1 (3,693) | 8.8 | 35.6 | 0.0 | 14 |
| LB and/or TBE[e] | | | | | |
| Asia | na[f] | na | na | na | 0 |
| Europe | 10.8 (2,047) | 9.7 | 23.0 | 0.0 | 13 |
| North America | 17.0 (393) | 7.5 | 35.6 | 5.3 | 5 |

*All the data presented come from published reports:
[a]References from Asia: Park *et al.*, 2003b; Walder *et al.*, 2006b;
[b]References from Europe: Brouqui *et al.*, 1995; Bakken *et al.*, 1996b; Dumler *et al.*, 1997; Fingerle *et al.*, 1997; Lebech *et al.*, 1998; Nuti *et al.*, 1998; Pusterla *et al.*, 1998b; Bjoersdorff *et al.*, 1999b; Christova & Dumler, 1999; Hunfeld & Brade, 1999; Cizman *et al.*, 2000; Oteo *et al.*, 2001; Skarphedinsson *et al.*, 2001; Daniel *et al.*, 2002; Groen *et al.*, 2002; Guillaume *et al.*, 2002; Zeman *et al.*, 2002b; Topolovec *et al.*, 2003; Walder *et al.*, 2003b; Grzeszczuk *et al.*, 2004b; Santino *et al.*, 2004; Tomasiewicz *et al.*, 2004; Cisak *et al.*, 2005; ; Kowalski *et al.*, 2006; Ongut *et al.*, 2006; Stanczak & Grzeszczuk, 2006;
[c]References from North America: Magnarelli *et al.*, 1995a, 1998; Pancholi *et al.*, 1995; Mitchell *et al.*, 1996; Fritz *et al.*, 1997; Wong *et al.*, 1997; Yeh *et al.*, 1997; Bakken *et al.*, 1998b; Wallace *et al.*, 1998; Hilton *et al.*, 1999; Ijdo *et al.*, 2000; Belongia *et al.*, 2001b; Aguero-Rosenfeld *et al.*, 2002; Leiby *et al.*, 2002;
[d]Includes adult and pediatric *B. burgdorferi* seropositive patients, farm workers, febrile patients, forest workers, patients with fever or undetermined origin, LB and TBE patients, individuals with tick bites or tick exposure;
[e]Includes adult and pediatric *B. burgdorferi* seropositive patients, LB and TBE patients;
[f]na, not available.

The occurrence of HGA is seasonal, with the majority of cases being reported between late spring and fall (McQuiston *et al.*, 1999; Ijdo *et al.*, 2000; Belongia *et al.*, 2001a; Blanco & Oteo, 2002; Gardner *et al.*, 2003; Lotric-Furlan *et al.*, 2006). In US, a bimodal distribution has additionally been demonstrated with a first peak of the disease in July through August and a second peak in October and November (CDC, 2001-2006). The seasonal pattern might result in part from the increase in outdoor activities during April-September, either recreational pursuits (e.g., camping, hiking, fishing, hunting, gardening, and walking dogs) or occupational activities, which involve an increased risk to tick exposure. Moreover, it also overlaps the period of the year when nymphal-stage of *Ixodes* ticks are most active. Even though both adults and nymphs are able to transmit *A. phagocytophilum*, the diminutive size of the latter stage allows them to often feed undetected on

humans long enough to transmit the pathogen. For this reason, it is currently believed that nymphs of the *Ixodes* genus are the main stage involved in *A. phagocytophilum* transmission to humans.

Despite the characteristically geographic and temporal pattern, HGA distribution might be influenced by changes in ecological or environmental conditions. Increased development in suburban and rural sites can result in an additional risk of tick exposure and local increases in HGA cases. Moreover, an impact from climate warming on the vertical disease distribution should be expected as has been suggested for other *Ixodes* borne-disease (Zeman & Bene, 2004; Ogden *et al.*, 2006).

Cases of HGA have been reported in both sexes and in different age groups from <1 to ≥90 years, although older male adults (median age 40-60 years) appear to be at higher risk for developing the disease (Gardner *et al.*, 2003; CDC, 2001-2006). Pediatric cases are restricted to only a few reports (Horowitz *et al.*, 1998c; Arnez *et al.*, 2001; Krause *et al.*, 2003; Moss & Dumler, 2003). This tendency is still not well understood and could be multifactorial. The rates of clinical infection could be related to age and/or gender specific increased susceptibility to recognizable disease and/or greater risk of exposure to *A. phagocytophilum*. Moreover, the nonspecificity of signs and symptoms that characterize HGA and the reluctance to draw blood, especially convalescent samples, from paediatric patients may contribute to fewer-than-expected enrolled children and, hence, underdiagnosis of HGA cases in children compared to adults. Two cross-sectional studies in endemic areas (Slovenia and Westchester County, New York) have indicated that up to 15% of children and younger adults (age, ≤24 years) have serologic evidence of previous exposure to *A. phagocytophilum* antigens, suggesting that infection might be more common than previously recognized (Cizman *et al.*, 2000; Aguero-Rosenfeld *et al.*, 2002).

Co-infections with *A. phagocytophilum* and other *Ixodes*-borne agents have occasionally been reported in humans, including *B. burgdorferi s.l.* and less frequently *Babesia* sp. in US and both *B. burgdorferi s.l.* and TBE virus in Europe (Nadelman *et al.*, 1997; Horowitz *et al.*, 1998a; Krause *et al.*, 2003; Moss & Dumler, 2003; Hermanowska-Szpakowicz *et al.*, 2004; Lotric-Furlan *et al.*, 2005, 2006; Grzeszczuk *et al.*, 2006b). Dual infections may result from a single tick bite and simultaneous transmission of different pathogens, from multiple tick bites or even from sequential infections that took place at different times (Levin & Fish, 2000, Des Vignes *et al.*, 2001; Belongia *et al.*, 2002). Although a rare event, coinfection should be taken into account in the clinical evaluation, diagnosis and treatment of patients potentially exposed to *Ixodes* ticks, a topic that will be further mentioned.

### 1.3.3. CLINICAL PRESENTATION

HGA is clinically variable ranging from mild self-limited flu-like illness, in the majority of cases, to severe multisystemic disease (Blanco & Oteo, 2002; Strle, 2004; Dumler, 2005). The undefined localization and nonspecific presentation defines a disease that is usually difficult to differentiate from many other infectious and non-infectious conditions.

Onset occurs after an incubation period of 1 to 2 weeks and clinical manifestations are dominated by fever, headache, myalgia and malaise (Aguero-Rosenfeld *et al.*, 1996; Bakken *et al.*, 1996c; Horowitz *et al.*, 1998a; Lotric-Furlan *et al.*, 2006). A minority of patients may also present with arthralgia or involvement of the gastrointestinal tract (nausea, vomiting, diarrhea and abdominal pain), respiratory tract (cough, pulmonary infiltrates), liver, or central nervous system. Despite the observation of stiff neck and other signs and symptoms that may suggest meningeal involvement, very little evidence supports invasion of the central nervous system by *A. phagocytophilum* (Dumler, 2005), although cases of lymphocytic meningitis have been described in coinfection with TBE (Grzeszczuk *et al.*, 2006b; Lotric-Furlan *et al.*, 2006). Some patients may present with conjunctivitis or lymphadenopathy, findings that are especially common in HGA European series (Laferl *et al.*, 1999; Arnez *et al.*, 2001; Karlsson *et al.*, 2001; Prukk *et al.*, 2003; Lotric-Furlan *et al.*, 2006). Local skin reactions at the site of the tick bite have not been described, and nonspecific skin-rashes have been reported only occasionally. The presence of erythema migrans has been documented in concurrent LB (Nadelman *et al.*, 1997; Horowitz *et al.*, 1998a; Grzeszczuk *et al.*, 2006b).

Variable changes in blood cell counts and chemistry parameters are reported (Hossain *et al.*, 1999; Bakken & Dumler, 2000; Bakken *et al.*, 2001; Lotric-Furlan *et al.*, 2006). Most patients develop transient depletion in platelet and total leukocyte (WBC) concentrations, usually accompanied by mild increases in serum hepatic transaminase concentrations (aspartate aminotransferase, alanine aminotransferase) or lactate dehydrogenase. Elevation of inflammatory markers, such as C-reactive protein and the erythrocyte sedimentation rate have also been found in the many HGA patients (Bakken *et al.*, 2001; Lotric-Furlan *et al.*, 2002). Other less common laboratory findings include anaemia, elevated serum creatinine and blood urea nitrogen levels. Although total WBC, absolute neutrophil, and lymphocyte counts may be below normal, patients with HGA often have a left shift during the first week of illness. Neutrophil counts gradually return to normal thereafter accompanied by relative and absolute lymphocytosis. The hematological

abnormalities tend to normalize by the end of the second week of illness, even in the absence of antimicrobial therapy. Thus, as stated by Bakken and Dumler (2000), caution is advised against dismissing HGA from the differential diagnosis if blood cell counts are normal at the time of presentation, especially for patients who have been ill for more than one week.

The results of physical examination are often unremarkable. Interestingly, several European cases of HGA have been presented as atypical pneumonitis, a fact that has also been mentioned in US literature (Bakken *et al.*, 1994, 1996c; Hardalo *et al.*, 1995; Lepidi *et al.*, 2000; Karlsson *et al.*, 2001; Remy *et al.*, 2003; Bayard-Mc Neeley *et al.*, 2004; Lotric-Furlan *et al.*, 2006; Mastrandrea *et al.*, 2006). Additionally, Halasz and coworkers (2005) have reported the association of HGA and Sweet Syndrome.

In regions where several *Ixodes*-borne diseases are known to occur, distinguishing between concurrent infections in the early stages of illness can be difficult. Infections with different agents can complicate disease presentation, and unusual signs, symptoms and laboratory abnormalities may be present. According to Krause and coworkers (2002), patients with LB and HGA and/or human babesiosis (HB)[6] experience more persistent flu-like symptoms with leukopenia and thrombocytopenia than those with LB alone. Acute LB is readily diagnosed when the pathognomonic erythema migrans is present, but some cases can lack that characteristic rash, overlapping HGA and HB clinical spectrum (Belongia *et al.*, 1999; Krause *et al.*, 2002). Likewise, HGA has a remarkably similar clinical and laboratory presentation to the initial phase of infection by TBE virus. However, prospective studies performed by Lotric-Furlan and coworkers (2000, 2002) have found statistically significant differences in the frequency of arthralgia and elevated concentration of C-reactive protein in patients with acute HGA than those with the initial phase of TBE. To sum up, the likelihood of coinfection should be considered when pursuing laboratory testing or selecting antimicrobial therapy.

### 1.3.4. COMPLICATIONS AND OUTCOME

HGA is usually an uncomplicated disease but nearly half of identified patients require hospitalization (Blanco & Oteo, 2002; Strle, 2004; Bakken & Dumler, 2006). In the US up to 17% of

[6] Human babesiosis is the general designation for infections caused by the tick-borne protozoan species *Babesia microti* and *B. divergens*. Cases of *B. microti* and *B. divergens* are chiefly reported from US and Europe, respectively.

patients are admitted to an intensive care unit (Bakken & Dumler, 2006). The severity of the disease appears to correlate with advanced age, immunosuppressive therapy, chronic inflammatory illnesses, or underlying malignant diseases (Bakken & Dumler, 2000). Delayed diagnosis and onset of specific antibiotic therapy are also risk factors for an adverse outcome. Severe complications have been described in the literature and are listed in table 8. Even though the fatality rate of HGA is low (<1%), at least 5 deaths have been identified in the US, three of which were the result of opportunistic infections, including exsanguination after ulcerative *Candida* esophagitis, ulcerative herpes simplex virus esophagitis with cryptococcal pneumonia, and invasive pulmonary aspergillosis (Bakken *et al.*, 1994; 1996c; Hardalo *et al.*, 1995; Walker & Dumler, 1997; Jahangir *et al.*, 1998; Lepidi *et al.*, 2000). As previously mentioned, this might result from the potential role of *A. phagocytophilum* in suppressing host defense or immune function. The risk of contracting HGA in immunocompromised patients, such as organ transplant recipients and persons infected with human immunodeficiency virus, is unknown. However, several cases have been described and severe manifestations may occur (Hardalo *et al.*, 1995; Adachi *et al.*, 1997; Trofe *et al.*, 2001; Vannorsdall *et al.*, 2002; Springer *et al.*, 2003; Bayard-Mc Neeley *et al.*, 2004). Regarding European cases, the disease appears to be generally milder and resolves sooner, even in the absence of specific antimicrobial therapy (Laferl *et al.*, 1999; Arnez *et al.*, 2001; Remy *et al.*, 2003; Lotric-Furlan *et al.*, 2006). Complications are unusual. No opportunistic infections have been described so far and only one case of fatal infection was reported in an article from Czech Republic (Hulinska *et al.*, 2002).

Ultimately, the immunosuppressive nature of HGA may also affect the clinical course of concurrent infection. Experimental studies have provided results with regard to the effect of *A. phagocytophilum* on *B. burgdorferi* dissemination, pathogen burden and host immune response (Thomas *et al.*, 2001; Holden *et al.*, 2005; Nyarko *et al.*, 2006). Nevertheless, the potential contribution of concurrent infections toward a more severe clinical course in humans remains to be defined. The available data is limited to few cases and in some instances are paradoxical. So far only two patients with TBE and HGA have been reported and both presented a relatively mild illness with uneventful recovery (Lotric-Fulan *et al.*, 2005, 2006). Regarding concurrent LB and HGA, although the diversity and duration of symptoms reported by patients with dual infections exceeds that for patients with LB alone, the frequencies of disseminated blood, skin, joint, cardiac, and neurologic disease and of hospital admission appear to be similar (Krause *et al.*, 2002).

TABLE 8. Clinical complications of HGA reported in North America and in Europe.

| Clinical Complications | Reference(s) |
|---|---|
| North America | |
| Acute abdominal syndrome | Greshel, 2000; Lepidi *et al.*, 2000 |
| Acute renal failure | Modi *et al.*, 1999 |
| Acute respiratory distress syndrome (ARDS) and pneumonitis | Bakken *et al.*, 1994; Hardalo *et al.*, 1995; Bayard-Mc Neeley *et al.*, 2004 |
| Carditis (pancarditis, pericarditis, myocarditis) | Goodman *et al.*, 1996; Jahangir *et al.*, 1998 |
| Coagulopathy and hemorrhage | Bakken *et al.*, 1994, 1996c |
| Neuropathies | Horowitz *et al.*, 1996; Bakken *et al.*, 1998a; Lee *et al.*, 2000 |
| Opportunistic infections | Bakken *et al.*, 1994, 1996c; Hardalo *et al.*, 1995 |
| Rhabdomyolysis | Shea *et al.*, 1996 |
| Septic or toxic shock-like syndrome | Bakken *et al.*, 1994 |
| Europe | |
| ARDS and pneumonitis | Bjoersdorff *et al.*, 1999a; Karlsson *et al.*, 2001; Tylewska-Wierzbanowska *et al.*, 2001; Remy *et al.*, 2003; Lotric-Furlan *et al.*, 2006 |
| Rhabdomyolysis | Van Dobbenburgh *et al.*, 1999 |

In general, the HGA outcome is favourable and disease resolves either after specific antibiotic therapy or even spontaneously (Lotric-Furlan *et al.*, 2001). Complete resolution of HGA can take only a few days, although biphasic courses with recurrent fever may occur in the absence of appropriate antibiotic therapy (Horowitz *et al.*, 1998d; Karlsson *et al.*, 2001; Walder *et al.*, 2003a, 2006a). This condition in humans seems to be rare but is not totally unexpected, since *A. phagocytophilum* is known to cause relapsing illness in ruminants (Tuomi, 1967 *in* Horowitz *et al.*, 1998d). In general, patients respond to treatment within 24 to 48 hours, but some with advanced infections may take longer or present persistent residual symptoms and signs, reflecting slow resolution of inflammation or accumulated tissue injury (Dumler, 2005).

Relapses or chronic infections have never been reported, even for those patients who were never treated with an active antibiotic. Reinfection, albeit rare, may occur, and was demonstrated in one US case (Horowitz *et al.*, 1998a). Persistence of *A. phagocytophilum* in humans is rarely observed in convalescent patients (Dumler & Bakken 1996; Ijdo *et al.*, 2000). Only two patients are known to have developed chronic sequelae following HGA (Horowitz *et al.*, 1996; Dumler & Bakken, 1998). Both patients received appropriate antibiotic therapy, and in each case, the irreversible neurological or tissue damage was believed to be caused by the acute inflammatory response elicited by the microorganism rather than by persistent infection. According

to Ramsey and coworkers (2002), some patients may also develop an apparently post-infectious syndrome, with persistent constitutional symptoms 1-3 years after treatment, such as fevers, fatigue, sweats, body pain, but without functional disability, but such conditions require much more study.

### 1.3.5. CONFIRMATORY LABORATORY TESTS

Confirmatory laboratory testing is required for a definitive diagnosis of HGA, as this is virtually impossible based only on epidemiologic, clinical and routine laboratory findings. Confirmatory methods include direct and indirect tests that allow the detection of *A. phagocytophilum* (microscopy, molecular analysis, *in vitro* culture) and antibodies elicited by infection (serological assays), respectively. The sensitivity of each confirmatory test regarding the disease onset is represented in figure 11.

Guidelines for HGA diagnosis have been published recently as the result of joint effort of epidemiologists, infectious disease clinicians, microbiologists, and pathologists to develop criteria for case definition and a consensus approach for laboratory testing (Walker *et al.*, 2000; Brouqui *et al.*, 2004).

#### 1.3.5.1. OPTICAL MICROSCOPY, MOLECULAR ANALYSIS AND *IN VITRO* CULTURE

Direct tests are useful to confirm HGA during active disease, prior to the initiation of effective antimicrobial therapy. Data based on observations of human and animal infection show that bacteremia occurs for a limited period of time that may persist for up two weeks or until the administration of effective antibiotic therapy, and diminishes rapidly to become undetectable thereafter (Bakken *et al.*, 1996c; Dumler, 2003). Since *A. phagocytophilum* infects granulocytic leukocytes, mostly mature neutrophils, direct tests are routinely performed in peripheral blood obtained at initial presentation to physician. Other available samples, including bone marrow aspirates and tissue specimens obtained at biopsy or autopsy may also be used. Blood-samples should be collected in ethylenediaminetetraacetic acid (EDTA) and kept refrigerated until processed, preferably within 24 to 48 hours. The use of heparin as an anticoagulant should be

avoided because of its ability to compromise PCR-based detection methods. Aseptic conditions for collection and preparation of samples is required if bacterial culture is to be done.

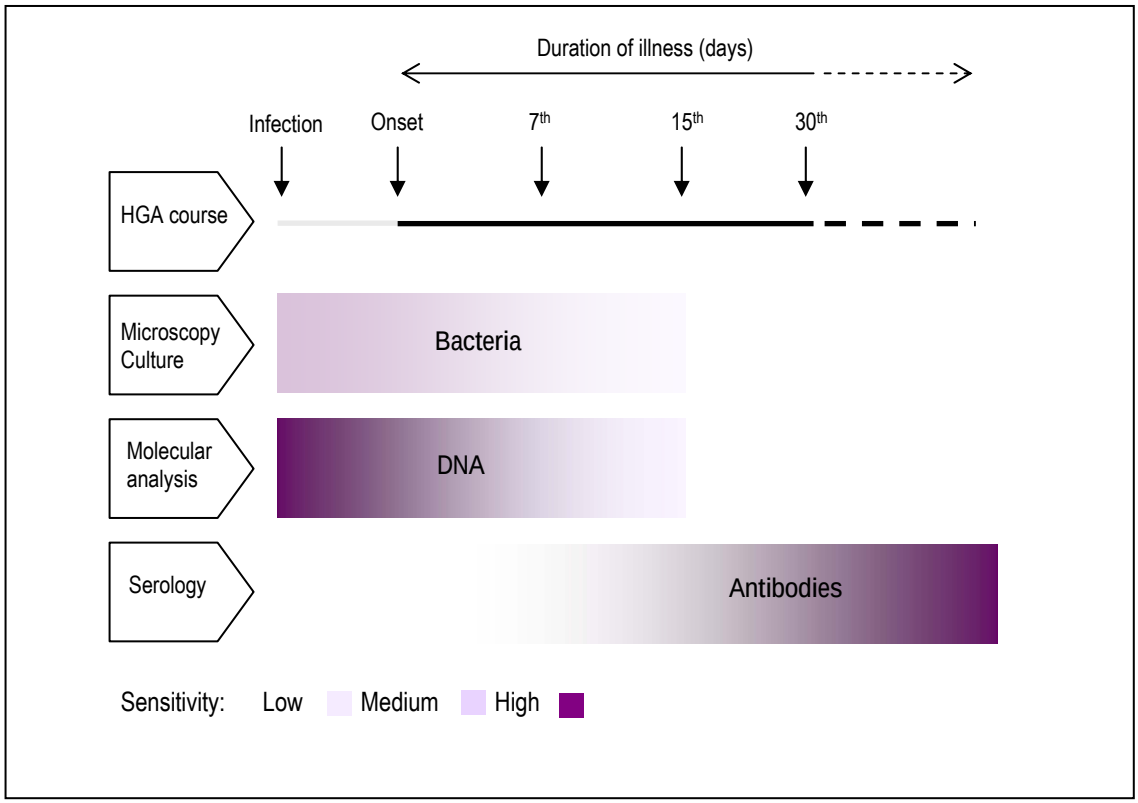



FIGURE 11. Relative sensitivity of diagnosis tests used for laboratory confirmation of HGA (Houpikian & Raoult, 2002; Bakken & Dumler, 2006).

Microscopic detection of *A. phagocytophilum* in blood is best achievable if smears are prepared immediately after sample collection. Buffy coat smears are preferred to regular whole-blood preparations, as most patients are leukopenic at the time of presentation and very few leukocytes are usually infected. Light microscopic examination of samples is performed under 400X or 1000X magnification and at least 800 to 1,000 granulocytes should be observed before considering a negative result (Aguero-Rosenfeld, 2000). The characteristic mulberry-shaped intraleukocytic morulae of *A. phagocytophilum* typically appear as dark blue to purple cytoplasmic densities after staining with eosin-azure (Romanovsky)-type dyes (including, Wright, Diff-Quik, Giemsa and Leishman stains) (Figure 12A). Although microscopy has been routinely applied for blood-smear testing, the examination of other available samples might also be useful. In severely ill patients, *A. phagocytophilum* morulae have been detected in bone marrow and other tissue samples, such as spleen, lung, liver and heart (Bakken *et al.*, 1994; Walker & Dumler, 1997;

Jahangir *et al.*, 1998; Lepidi *et al.*, 2000; Trofe *et al.*, 2001; Remy *et al.*, 2003; Bayard-Mc Neeley *et al.*, 2004). The examination of bone marrow smears should be performed after sample staining with eosin-azure (Romanovsky)-type dyes (Remy *et al.*, 2003; Bayard-Mc Neeley *et al.*, 2004). Other tissue specimens can be tested either fresh, following formalin fixation and paraffin embedding, using immunohistochemistry techniques (Figure 12B) (Bakken *et al.*, 1994; Walker & Dumler, 1997; Lepidi *et al.*, 2000).

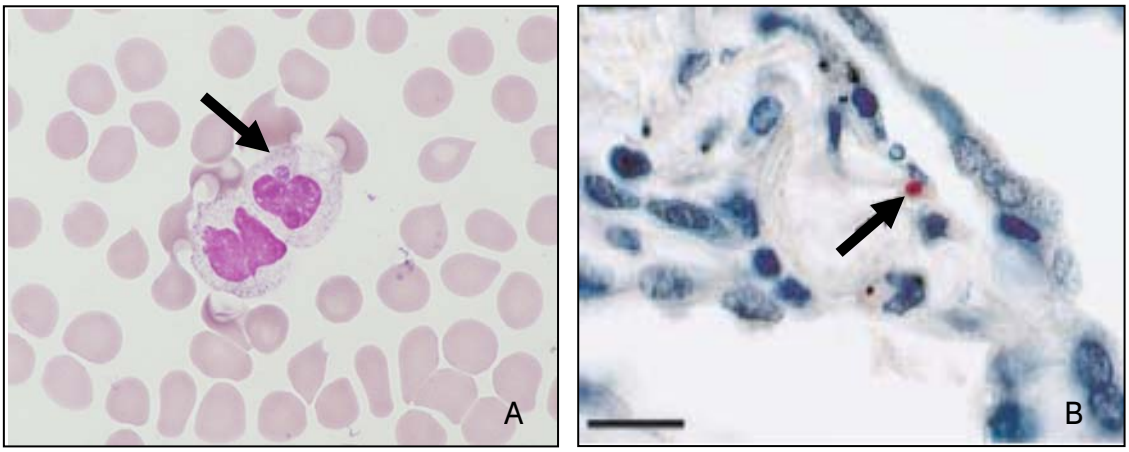



FIGURE 12. *A. phagocytophilum* morulae in neuthrophils: A- pheripheral blood smear stained by Diff-Quik; arrows- infected neutrophils, 1000X magnification (courtesy of JS Dumler); B- spleen section stained by immunoalkaline phosphatase/ hematoxylin; arrows- infected neutrophils within splenic red pulp sinusoids, bar- 20 μm (adapted from Lepidi *et al.*, 2000).

Observation of blood smears is the most inexpensive and fastest laboratory test, but is also the least sensitive. *A. phagocytophilum* morulae may be sparsely distributed and difficult to detect, even by experienced observers and a negative result should not rule out HGA. Case series describing US patients with culture-confirmed HGA report only 60% morulae visualization, and although as much as 40% of peripheral granulocytes may contain *A. phagocytophilum* inclusions, the majority of patients with positive smears had less than 1% infected granulocytes (McQuiston *et al.*, 1999; Bakken *et al.*, 2001). In Europe the detection of *A. phagocytophilum* in peripheral-blood smears seems to be an infrequent finding, observed only in four patients (Van Dobbenburgh *et al.*, 1999; Remy *et al.*, 2003; Beltrame *et al.*, 2006; Garcia *et al.*, 2006) (Table 9). Moreover, false positive interpretations may also occur due to toxic granulations, Döhle bodies, or superimposed platelets, contaminant particles, or other inclusions which may be mistaken for organisms. Thus, microscopic examination of samples should be always conducted in parallel with other tests to confirm HGA diagnosis (Walker, 2000; Brouqui *et al.*, 2004).

TABLE 9. Results of confirmatory laboratory testing for 330 patients with HGA from case series or case reports in North America[†] and Europe[‡].

| Confirmatory test | No. (%) patients with positive test result | | Total |
|---|---|---|---|
| | North America[†] n=277 | Europe[‡] n=53 | |
| Morulae in neutrophils | 116/238 (48.7) | 4/43 (9.3) | 120/281 (42.7) |
| Positive PCR result | 112/185 (60.5) | 31/47 (65.9) | 143/232 (61.6) |
| Isolation of *A. phagocytophilum* in cell-culture | 39/64 (60.9) | - | 39/64 (60.9) |
| Seroconversion or ≥four-fold change in antibody titer[a] | 232/273 (85.0) | 50/58 (86.2) | 282/331 (85.2) |

[†]References of 277 HGA patients (representing 266 confirmed and 11 probable cases): Aguero-Rosenfeld *et al.*, 1996, 2000; Goodman *et al.*, 1996; Horowitz *et al.*, 1998b; Wallace *et al.*, 1998; Belongia *et al.*, 1999; Foley *et al.*, 1999a; Bakken *et al.*, 2002;
[‡]References of 53 HGA patients: Laferl *et al.*, 1999; Van Dobbenburgh *et al.*, 1999; Oteo *et al.*, 2000; Arnez *et al.*, 2001; Karlsson *et al.*, 2001; Kristiansen *et al.*, 2001; Tylewska-Wierzbanowska *et al.*, 2001; Bjoersdorff *et al.*, 2002b; Prukk *et al.*, 2003; Remy *et al.*, 2003; Rusio & Cinco, 2003; Walder *et al.*, 2003a, 2006a; De la Fuente *et al.*, 2005b; Beltrame *et al.*, 2006; Garcia *et al.*, 2006; Grzeszczuk *et al.*, 2006b; Lotric-Furlan *et al.*, 2006; Mastrandrea *et al.*, 2006; Misić-Majerus *et al.*, 2006;
[a]Indirect immunofluorescence assay for acute- and convalescent-phase samples.

The detection of *A. phagocytophilum* DNA is now becoming the standard complement for serologic assays (Dumler 2003; Brouqui *et al.*, 2004). As for other direct tests, the most appropriate sample for molecular analysis is EDTA-anticoagulated whole-blood or buffy coat, although the usefulness of other available samples, such as serum, cerebrospinal fluid (CSF), and skin biopsies, has been described (Massung *et al.*, 1998; Comer *et al.*, 1999b; Lee *et al.*, 2000; Halasz *et al.*, 2005). Several primer sets are described in the literature for the amplification of specific nucleic acid sequences of *A. phagocytophilum* by PCR in simple or nested reactions (Table 10). Among the targeted genes are the *rrs*, *groESL*, *ankA*, *msp2* (*p44*) (Chen *et al.*, 1994; Pancholi *et al.*, 1995; Goodman *et al.*, 1996; Sumner *et al.*, 1997; Massung *et al.*, 1998; Walls *et al.*, 2000; Caspersen *et al.*, 2002). However, PCR-based methods are not yet standardized and may yield discrepant results. Sensitivity might be dependent on sample quality, target sequence (that may have multiple copies in *A. phagocytophilum* genome, as in the case of *ankA* and *msp2* and in the amplicon length (Dumler, 2003; Brouqui *et al.*, 2004). The detection limits of 13 published PCR assays have been assessed, and those targeting fragments of the outer surface proteins homologues or *rrs* were found to be the most sensitive (Massung & Slater, 2003), but this study was conducted prior to the availability of many other gene targets, application of real time PCR, and the publication of the genome sequence. Whichever DNA target is chosen, sequencing of PCR products is strongly encouraged to confirm their identity.

TABLE 10. DNA targets that have been used for the detection of *A. phagocytophilum* in samples from infected patients.

| Target gene | Primer pair | PCR method/ amplified fragment | Detection Limit/ Specificity† | Reference |
|---|---|---|---|---|
| *ankA* | LA1/LA6 | regular/ 444-bp | 2.5/ – | Walls *et al.*, 2000 |
| *groESL* | HS1/HS6; HS43/HS45 | nested/ 442-bp | 2.5/ Ec | Sumner *et al.*, 1997 |
| *msp2 (p44)* | MSP465f/MSP980r | regular/ 550-bp | NT | Caspersen *et al.*, 2002 |
| *rrs* | GE9f/GE10r | regular/ 919-bp | 2.5/ – | Chen *et al.*, 1994 |
| | Ehr521/Ehr747 | regular/ 247-bp | 0.25/ Bh, Ec, Rk | Pancholi *et al.*, 1995 |
| | Ehr521/Ehr790 | regular/ 293-bp | 0.25/ Ec | Kolbert, 1996 |
| | PER1/PER2 | regular/ 451-bp | 2.5/ Ec | Goodman *et al.*, 1996 |
| | GER3/GER4 | regular/ 151-bp | NT | Goodman *et al.*, 1996 |
| | GE3a/GE10r; GE9f/GE2 | nested/ 546-bp | 0.25/ – | Massung *et al.*, 1998 |
| | EC9/EC12; GE9f/GE10r | nested/ 919-bp | 2.5/ – | Chen *et al.*, 1994 |

†According to Massung & Slater, 2003:
Detection limit was estimated by the minimum number of *A. phagocytophilum* infected cells that could be detected per test sample. Sensitivity was detected using template DNA from *Bartonella henselae* strain Houston-1 (Bh), *Ehrlichia chaffeensis* strain Arkansas (Ec), and *Rickettsia rickettsii* strain Sheila Smith (Rk);
NT- not tested; – no detection of *B. henselae, E. chaffeensis* or *R. rickettsii*, DNA.

Since the first report of *A. phagocytophilum* isolation in HL-60 cells by Goodman and coworkers (1996), several investigators have used this modality of direct HGA diagnosis. Cells are maintained in antibiotic-free RPMI-1640 medium, supplemented with 2 mM glutamine and fetal bovine serum 5% v/v, and incubated at 37ºC in an atmosphere of 5% $CO_2$. For isolation assays, 0.1 to 0.5 ml anticoagulated whole-blood or buffy coat is inoculated straight into HL-60 cells that are maintained at a final concentration of $2x10^5$ cells/ml. Cultures are evaluated every 2 to 4 days to adjust cell concentration and detect any microbial growth. Infection can be assessed by direct observation of cytocentrifuged culture aliquots after staining by eosin-azure type dyes. To date, almost all human isolates of *A. phagocytophilum* came from US patients, with the exception of one isolate obtained from a Slovenian patient (JS Dumler, personal communication), and in the majority of cases they were obtained from blood-samples. Only two reports describe the isolation of *A. phagocytophilum* from CSF and bone marrow samples (Lee *et al.*, 2000; Bayard-Mc Neeley *et al.*, 2004). According to Aguero-Rosenfeld (2002), most of the cultures from patients showing morulae on blood smears become positive during the first week of incubation, and for patients with negative smears it can take two weeks or more. Culturing of *A. phagocytophilum* is regarded as the most specific method to confirm the diagnosis of HGA although it is still not widely available. Only a

limited number of research institutions are currently able to perform culture assays. Even so, this might not be a constraint; Kalantarpour and coworkers (2000) have showed that *A. phagocytophilum* can remain viable in infected blood kept for up 18 days under refrigerated conditions thus biological samples can be easily transported to a referral laboratory where the appropriate assays can be carried out. The only limitation is that cultures will require more time to become positive (Kalantarpour *et al.*, 2000).

#### 1.3.5.2 SEROLOGY

In most cases definitive HGA diagnosis relies on the demonstration of a serologic response to *A. phagocytophilum*. Indirect immunofluorescence assay (IFA) is currently the best overall option. Other serological methods include enzyme immunoassay and immunoblotting methods that employ purified antigens or recombinant proteins, but to date no standardization of these tests exists, and all are performed at the research level.

IFA testing is performed with serum or plasma samples, using *A. phagocytophilum* strains as a source of antigen, and most laboratories detect IgG and IgM separately, or total immunoglobulins. Antibody titer values are expressed as the reciprocal of the representative serum dilution at which unequivocal apple-green fluorescence of microbial inclusion bodies is focally located in the cytoplasm of the infected cells (Figure 13). Slide examination is performed in an UV microscope under 400X or preferably 1000X magnification. The cut-off value should be determined in each laboratory based upon well-characterized sera from patients with proven HGA and an uninfected control population (Walker *et al.*, 2000). Samples reactive at a dilution of 1:64 or 1:80 are usually considered positive (Dumler *et al.*, 1995; Bakken *et al.*, 1998b), and serial dilutions are done to determine the end-point titer.

In general, IFA testing of acute and convalescent-phase samples is the most sensitive laboratory tool for diagnosis confirmation of HGA (Table 9). The lack of humoral response has been described only in few cases and is still not well-understood (Aguero-Rosenfeld *et al.*, 1996, 2000; Bakken *et al.*, 2002; De la Fuente *et al.*, 2005b, Lotric-Furlan *et al.*, 2006). One possible explanation is that early therapy may abrogate antigenic mass sufficiently to preclude a detectable antibody response (Aguero-Rosenfeld *et al.*, 2000; Bakken *et al.*, 2002).

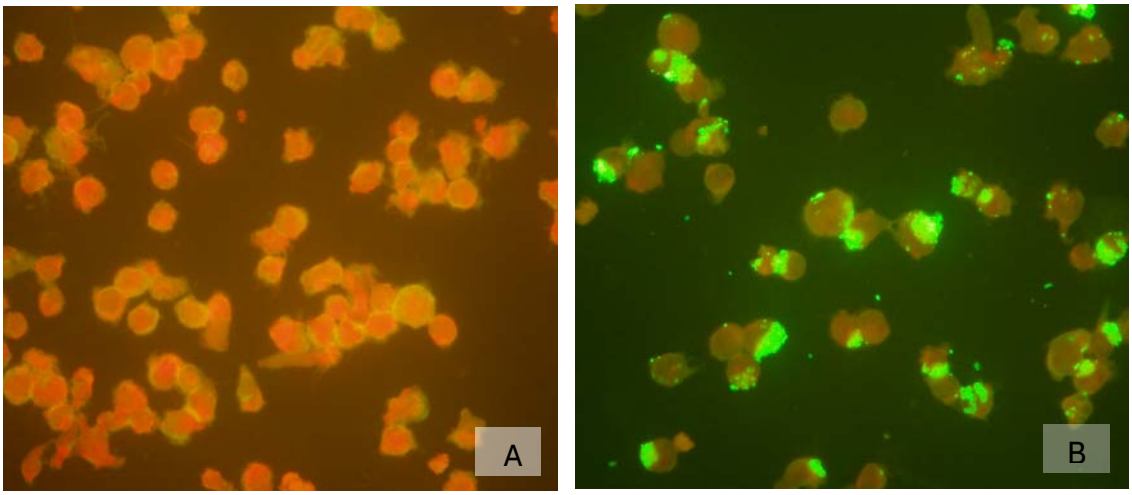



FIGURE 13. Immunofluorescence assay (IFA) using HL-60 cells infected with *A. phagocytophilum* Webster strain as antigen, 1000X magnification: A- negative sample; B- reactive sample evidenced by green fluorescence of *A. phagocytophilum* morulae.

The major disadvantage of IFA is that diagnosis confirmation is usually achieved retrospectively. Antibodies usually appear within two weeks after the onset of disease, reaching the highest titer during the first month of illness, and may remain detectable for a year or more (Aguero-Rosenfeld *et al.*, 2000; Lotric-Furlan *et al.*, 2001; Bakken *et al.*, 2002). Therefore, a negative serologic result in an acutely ill patient is typical and does not exclude HGA. Diagnostic confirmation usually depends on the availability of paired serum samples from acute and convalescent phases (obtained 2 to 4 weeks apart) to show seroconversion or a four-fold increase in antibody titer. Several studies have showed that only 20-40% of patients with confirmed HGA had antibodies detected by IFA at the time of initial presentation to the physician; however, more than 90% patients developed titers exceeding the cut-off within the first month of illness (Comer *et al.*,1999a; Aguero-Rosenfeld *et al.*, 2000; Lotric-Furlan *et al.*, 2001; Bakken *et al.*, 2002).

Another limitation of IFA testing is cross-reactivity that might be present between antigens from other pathogens not only in the same genus but also from different genera. Cross-reactivity between *A. phagocytophilum* and *E. chaffeensis* is suspected to occur in approximately 10-20% of patients tested (Wong *et al.*, 1997; Walls *et al.*, 1999; Comer *et al.*, 1999a). In these circumstances, serologic differentiation is best accomplished by simple comparison of antibody titers, since higher titers are usually developed to the homologous microorganism. Simultaneous testing for antibodies to both agents is advised to differentiate between the two diseases,

especially in regions where both pathogens are present. The diagnosis of HGA or HME would depend on the presence of a four-fold or greater titer against *A. phagocytophilum* or *E. chaffeensis*, respectively. Although less frequent, cross-reactivity with other agents such as *B. burgdorferi s.l.*, *C. burnetii*, and *Rickettsia* spp. has also been described (Comer *et al.*, 1999a; Blanco & Oteo, 2002). Moreover, patients with Q fever and bartonellosis have been described to react with *A. phagocytophilum* antigen (Brouqui *et al.*, 2001). Whether these findings represented cross-reactivity directed to common antigens, such as the heat shock proteins, or antibody induced by separate exposures to different organisms remains to be ascertained (Wong *et al.*, 1997; Ijdo *et al.*, 1998b; Unver *et al.*, 2001a). At least in some cases, *B. burgdorferi* and TBE virus serologic cross-reactions with *A. phagocytophilum* have been explained by confirmed coinfections with both agents (Nadelman *et al.*, 1997; Horowitz *et al.*, 1998a; Belongia *et al.*, 1999; Bjoersdorff *et al.*, 2002b; Moss & Dumler, 2003; Lotric-Furlan *et al.*, 2005, 2006; Grzeszczuk *et al.*, 2006b).

The problem of potential cross-reactivity of antibodies and the possibility of dual or past infections indicate that additional testing is required to confirm simultaneous HGA. Another way to distinguish cross-reactivity is by use of immunoblots demonstrating reactivity to specific antigens. As mentioned by Dumler (2003), several recombinant proteins- including members of the Msp2 (P44) major outer surface protein family, AnkA, and GroEL - have been cloned and evaluated as potential serodiagnosis reagents with variable promise (Ijdo *et al.*, 1998a Murphy *et al.*, 1998; Storey *et al.*, 1998; Zhi *et al.*, 1998; Caturegli *et al.*, 2000).

#### 1.3.5.3. CASE DEFINITION

The diagnosis of HGA requires the combination of an adequate history of tick exposure, suggestive clinical and laboratory findings, such as documented fever in the absence of specific findings during physical examination, abnormal hematological/ metabolic values and diagnosis confirmation by use of one or several specific laboratory tests.

The criteria for case definition of confirmed and probable infection by *A. phagocytophilum* are showed in table 11.

TABLE 11. Proposed case definition for human granulocytic anaplasmosis (adapted from Brouqui *et al.*, 2004).

| |
|---|
| Confirmed infection |
| Febrile illness with a history of a tick bite or exposure to tick-infested habitats<br>*and*<br>Demonstration of seroconversion or ≥four-fold change in antibody titer in acute and convalescent samples[a]<br>*or*<br>Positive PCR result with subsequent sequencing of the amplicons demonstrating *Anaplasma*-specific DNA[b]<br>*or*<br>Isolation of *A. phagocytophilum* in culture |
| Probable infection |
| Febrile illness with a history of a tick bite or exposure to tick-infested habitats<br>*and*<br>Presence of a stable antibody titer in acute and convalescent samples if titer >four-fold above the cut-off value[c]<br>*or*<br>Positive PCR result without sequence confirmation[d]<br>*or*<br>Presence of intracytoplasmic morulae in neutrophils |

[a]By indirect immunofluorescence antibody test with *A. phagocytophilum* antigen; [b]The American Society of Microbiology's Task Force on Consensus Approach for Ehrlichiosis considers positive PCR alone (without other laboratory support) to represent probable laboratory evidence of HGA (Walker *et al.*, 2000); [c]A cut-off value of 1:80 is currently used; [d]With *A. phagocytophilum* species-specific primers.

### 1.3.6. TREATMENT

Since it is difficult to predict whether patients with *A. phagocytophilum* infection will experience a mild or severe clinical course, prompt institution of antibiotic therapy is advocated for all individuals who are symptomatic and suspected of having HGA (Bakken & Dumler, 2006; Chapman *et al.*, 2006; Wormser *et al.*, 2006a). Specific laboratory tests that confirm clinical suspicion are generally not available in the acute care setting, making empiric antibiotic therapy necessary pending the results of confirmatory tests. Table 12 shows the recommended antibiotic therapy for HGA.

Tetracyclines have been traditionally considered the first-line antibiotics for treatment of HGA, based on clinical efficacy and uniform *in vitro* susceptibility of *A. phagocytophilum* strains for these compounds (Bakken *et al.*, 1994; Dumler & Bakken, 1995; Aguero-Rosenfeld *et al.*, 1996; Bakken *et al.*, 1996c; Walker & Dumler, 1996; Klein *et al.*, 1997; Dumler & Bakken, 1998; Horowitz *et al.*, 2001; Maurin *et al.*, 2003; Branger *et al.*, 2004). Doxycycline hyclate is the treatment of

choice due to its excellent pharmacokinetic properties and less frequent gastric intolerance compared with other tetracycline derivatives (Bakken & Dumler, 2006). It also has the advantage of showing additional activity against *B. burgdorferi* (Bakken & Dumler, 2006; Wormser *et al.*, 2006a), covering potential coinfections in areas where both LB and HGA are endemic. Although broad-use of tetracyclines derivatives is typically contraindicated during pregnancy, its use is advocated in life-threatening situations (Chapman *et al.*, 2006). Moreover, the American Academy of Pediatrics Committee on Infectious Diseases has revised its recommendations in 1997 and precluded limited doxycycline courses that do not pose a substantial risk for tooth staining, as the preferred antibiotic treatment for children of any age diagnosed with clinically apparent HGA, RMSF or HME (Chapman *et al.*, 2006). In fact, doxycycline therapy has been successfully used for treatment of a newborn with HGA (Horowitz *et al.*, 1998c) and a 5-year-old child, who had simultaneous LB (Moss & Dumler, 2003).

TABLE 12 – Recommended adult and pediatric antibiotic treatment for HGA (adapted from Bakken & Dumler, 2006; Wormser *et al.*, 2006a).

| Antibiotic | Dose[a] | | Duration (days) |
|---|---|---|---|
| | Adults | Children | |
| Doxycycline hyclate[b] | 100 mg i.v. or p.o.q 12h[c] | 2 mg/Kg p.o.q 12h (max. 100 mg/dose)[d] | 5-14 |
| Rifampin[e] | 300 mg p.o.q 12h | 10 mg/Kg p.o.q 12h (max. 300 mg/dose) | 7 |

[a]i.v. Intravenous administration, p.o. Oral administration; [b]Ineffective for HB; [c]A treatment of 10 days is usually recommended for adults with HGA or 14 days if coinfection with *B. burgdorferi* is suspected; [d]Children ≥8 years-old may be treated with a 10-day course. Children >8 years-old are recommended to receive an abbreviated treatment of 4–5 days (i.e., for 3 additional days after resolution of fever). If concomitant LB is suspected, administration of amoxicillin (50 mg/kg per day in 3 divided doses [maximum of 500 mg per dose]) or cefuroxime axetil (30 mg/kg per day in 2 divided doses [maximum of 500 mg per dose]) is recommended at the conclusion of doxycycline treatment to complete a 14-day total course of antibiotic therapy; [e]Ineffective therapy for LB and HB.

*In vitro* studies also showed that rifamycins have excellent activity against *Anaplasma* species (Klein *et al.*, 1997; Horowitz *et al.*, 2001; Maurin *et al.*, 2003; Branger *et al.*, 2004). This group of antibiotics is regarded as an alternative therapy for patients in whom tetracyclines use are contraindicated or limited, such as pregnant or lactating women, children younger than 8 years of age if not seriously ill, and intolerant or allergic individuals. Rifampin has already been used successfully in a small number of pediatric patients and pregnant women (Buitrago *et al.*, 1998; Dumler & Bakken, 1998; Krause *et al.*, 2003). However, the use of rifampin should be considered

with caution because of the possibility of rapid selection of resistant microbial populations, as has been demonstrated for other bacterial species [(Enright *et al.*, 1998; Heep *et al.*, 1999) *in* Maurin et al, 2003]. Other compounds such as fluoroquinolones have also showed *in vitro* activity against *A. phagocytophilum*, although further clinical data is needed to define a safe alternative to tetracyclines (Klein *et al.*, 1997; Horowitz *et al.*, 2001; Maurin *et al.*, 2003; Branger *et al.*, 2004). For example levofloxacin, one of the most active fluoroquinolones against *A. phagocytophilum*, has been recently implicated in poor *in vivo* activity with the report of a clinical and microbiological relapse after completion of treatment (Wormser *et al.*, 2006b). Moreover, a *gyrA*-mediated resistance in the related species *E. canis* and *E. chaffeensis* that likely corresponds to a single amino acid difference in the GyrA protein in *A. phagocytophilum*, has recently been described (Maurin *et al.*, 2001).

*In vitro* investigations of several clinical isolates have demonstrated that *A. phagocytophilum* is resistant to the majority of broad-spectrum antimicrobials such as β-lactam compounds (ampicillin, amoxicillin, ceftriaxone), macrolides (azithromycin, clarithromycin, erythromycin, telithromycin), amikacin, sulfamethoxazole-trimethoprim and chloramphenicol (Klein *et al.*, 1997; Horowitz *et al.*, 2001; Maurin *et al.*, 2003; Branger *et al.*, 2004). The lack of a peptidoglycan layer or LPS in *A. phagocytophilum* outer membrane (Lin & Rikihisa, 2003; Hotopp *et al.*, 2006) is a possible explanation for β-lactam resistance. This situation is similar to that of mycoplasmas, which are also naturally resistant to all β-lactam compounds (McCormack, 1993 *in* Branger *et al.*, 2004). Macrolide resistance is probably the result of point mutations that have been observed in *A. phagocytophilum* 23S rRNA genes, and are known to confer resistance in other bacteria (Vester & Douthwaite, 2001 *in* Maurin et al, 2003; Branger *et al.*, 2004). Amoxicillin, azithromycin, and erythromycin, as well as the cephalosporin ceftriaxone, are agents that may be used to treat *B. burgdorferi* infection (Wormser *et al.*, 2006a). Thus, persistent symptoms or slow resolution of LB after treatment with β-lactam or macrolides antibiotics may suggest underlying HGA. The same is applicable for HB that uses atovaquone in combination with azithromycin as one of the treatment options (Wormser *et al.*, 2006a).

A close clinical follow-up is recommended to ensure that patients respond as expected to therapy. Although severely ill individuals might require longer periods before clinical improvement is noted, especially if they have dysfunction of multiple organs, administration of effective HGA therapy usually results in defervescence within 24 to 48 hours (Bakken *et al.*, 1994;

Dumler & Bakken, 1995; Aguero-Rosenfeld *et al.*, 1996; Bakken *et al.*, 1996c; Walker & Dumler, 1996; Buitrago *et al.*, 1998; Dumler & Bakken, 1998; Horowitz *et al.*, 1998c; Krause *et al.*, 2003; Bakken & Dumler, 2006). Thus, patients who fail to respond to treatment within this time frame should be reevaluated for alternative diagnosis, including the possibility of coinfections. Although tetracyclines are active against *B. burgdorferi* but not *Babesia* spp., rinfampin is inactive against both (Wormser *et al.*, 2006a).

### 1.3.7. PREVENTION

The best currently available method for preventing infection with *A. phagocytophilum*, as for other tick–borne pathogens, is to avoid tick exposure. Strategies aimed to reduce vector densities in backyards and regular application of ectoparasite control on pets help to control infestations of domestic environments. Moreover, the use of both protective clothing and tick repellents when entering tick-infested habitats are also important measures recommended to reduce the risk of infection.

Long-sleeved shirts tucked into pants, long pants tucked into socks, and closed-toe shoes are helpful in preventing ticks from reaching the skin and attaching. The selection of khaki neutral-colored clothing is preferred to allow a background contrast with any eventual crawling ticks. Various over-the-counter products containing DEET (*N,N*-diethyl-*m*-toluamide) are available for topical application on exposed skin. The optimal concentration ranges from 15%-35%, which allows high performance and a high margin of safety. There is no evidence that concentrations >50% are more efficient or provide longer protection period. The timing of reapplication depends on the specific preparation utilized. Some products may provide up to 12 hours protection from one application, but long-acting formulations are being developed (Salafsky *et al.*, 2000). Neurologic manifestations in children after excessive application of DEET-containing repellents have been reported (CDC, 1989), but the compound appears to be safe when used as directed in the product labels, even for young children 12 months old (Fradin, 1998; Qiu *et al.*, 1998; Koren *et al.*, 2003). Products containing Permethrin can be used to treat outer clothing (e.g., shirts and pants). It should be applied according to label directions in a well-ventilated area and clothing must completely dry before being worn. This chemical is nontoxic to humans although it is not licensed for direct application to the skin.

In tick-infested habitats it is also important to avoid wooded or grassy areas and walk on cleared trails. Brushing against tall grass and other vegetation is regarded as a risk factor (Lane *et al.*, 2004). This practice is particularly essential during periods of peak tick activity (i.e., late spring and summer) but should be followed throughout the year, regardless of the season. Frequent visual inspection of skin and clothes may help to identify crawling ticks preventing attachment. Limited data exist regarding the interval of *A. phagocytophilum* transmission after tick attachment, but animal studies indicate that 24-48 hours might elapse before effective pathogen transmission (Hodzic *et al.*, 1998; Katavolos *et al.*, 1998; Des Vignes *et al.*, 2001); transmission has been demonstrated under experimental circumstances in as little as 4 hours after attachment. The risk of *A. phagocytophilum* infection and other tick borne-diseases can therefore be reduced substantially by performing daily inspections of entire body and by promptly removing attached ticks after outdoor activities.

Attached ticks should be removed preferably by grasping with tweezers or fine-tipped forceps close to the skin and gently pulling in a constant and steady motion. Folk methods of removing ticks, such as using lighted cigarettes, petroleum jelly, fingernail polish, etc, should be avoided because they may increase the risk of agent transmission by increasing tick salivation or body damage and consequent fluid leaking into the wound (Needham, 1985; Boer & Van den Boggard, 1993). If a portion of the ticks mouth parts remains embedded in the skin, only topical disinfection of the site is suggested, because attempts to remove this material can cause tissue damage and are unnecessary as the risk of agent transmission is unaffected. Ticks that have been removed should not be crushed between the fingers to prevent contamination. After tick removal, the bite wound should then be disinfected and hands should be washed to avoid potential conjunctival contamination. Although there is a potential risk of infection, most tick bites are uncomplicated and result only in benign cutaneous inflammatory reactions that may be pruritic for a few days. Sometimes a granuloma may develop, supposedly as a result of mouthparts being retained at the feeding site (Parola & Raoult, 2001a).

The prophylactic potential of antimicrobials have been studied in animal model of granulocytic anaplasmosis (Blagburn *et al.*, 2004; Massung *et al.*, 2005b) but its usefulness for humans has not been demonstrated. In general, the use of antibiotics is strictly recommended for clinically ill individuals.

## 1.4. ANAPLASMATACEAE IN PORTUGAL

The presence of Anaplasmataceae family member species in Portugal has been long recognized starting with the 1943 description of bovine and ovine anaplasmosis due to *A. marginale* (Silva Leitão, 1943). Today the disease is still regarded as a veterinary problem that affects flocks and herds across the country, with the highest prevalence found in Alentejo region, Southern mainland Portugal (J Gomes, personal communication).

In 1990, Filipe and coworkers described an *Ehrlichia*-like organism in the hemolymph of *Rhipicephalus sanguineus* ticks parasitizing a domestic dog from Setúbal District (Filipe *et al.*, 1990). The agent was later maintained in a *R. sanguineus*-laboratory reared colony by intracelomic inoculations and found to be antigenically similar to *E. canis* (Santos, 1997). Additional studies, report that *E. canis* seroprevalence ranges from 45.4 - 54.5% in stray dogs from this same geographic area (Silveira, 1992; Bacellar *et al.*, 1995). More recently, *E. canis* DNA was detected in a dog hospital population in the Algarve region, Southern mainland, definitively confirming the association of this Anaplasmataceae family member with canine disease in Portugal (Alexandre, 2006). Due to the association of *E. canis* and *R. sanguineus*, one of the most prevalent tick species in the region (Caeiro, 1999), it is expected that canine monocytic ehrlichiosis occurs broadly across the country, especially in the Southern regions.

The existence of other Anaplasmataceae member species and their potential involvement in human disease was first suggested by David de Morais and coworkers (1991). The authors described a case of febrile illness without rash, accompanied by leucopenia, thrombocytopenia, mild anemia and increase of hepatic enzymes in a 21-year-old man from Évora District, Alentejo region. The patient worked in an office and had no regular contact with animals and no history of tick bite, although he had visited a farm two weeks before the onset of symptoms in January 1991. Serological tests for typhoid fever, brucellosis, Mediterranean spotted fever (MSF), and LB were negative. Human monocytic ehrlichiosis was suggested by a >four-fold decrease in IFA titer against *E. chaffeensis* antigen in two consecutive samples collected 15 days after the onset and two months later, showing an antibody decrease from 1024 to 128. Three months after the onset of disease, the serum titer against *E. chaffeensis* was 64. A high, stable IFA titer of 512 against *C. burnetii* phase II antigen was also observed in all three serum samples (David de Morais *et al.*, 1991, 1992b). Based on serology, another patient with a febrile illness associated with *E*

*chaffeensis* antibody was described by Rodrigues and coworkers (1998). However, neither agent isolation nor convincing DNA detection have been achieved in human, animal, or tick samples to reinforce these data.

Additionally, a study performed in Madeira Island designed to clarify the importance of *I. ricinus* as vector of tick-borne agents showed for the first time the presence of *A. phagocytophilum* DNA in this tick species (Núncio *et al.*, 2000). The finding was not completely unexpected since the agent was long known to occur in Europe (Gordon *et al.*, 1932) and had been previously detected in *I. ricinus* from another Mediterranean region (Cinco *et al.*, 1997) but this observation highlighted the possible role of Anaplasmtaceae in Public Health, and added to the list of *Ixodes*-borne agents in Portugal potentially involved in human diseases.

*I. ricinus* is described as a hygrophilic tick [(Daniels *et al.*, 1977; Gray, 1991; EUCALB) *in* Baptista, 2006] dispersed throughout Portugal with a patchy geographic distribution mainly influenced by environmental determinants such as climate (temperature, humidity and precipitation), landscape fragmentation, and landscape composition (open areas, mixed and deciduous forests) (Baptista, 2006). Although it has been reported from all districts of mainland Portugal (Dias *et al.*, 1994; Caeiro, 1999), when compared with other species such as *R. sanguineus* and *Dermacentor marginatus*, it has a more restricted distribution, with the highest prevalence registered in rural regions (Baptista, 2006). Moreover, *I. ricinus* is also present in the Atlantic Archipelagos of Madeira and Açores, essentially in Madeira and Pico Islands, respectively [(Dias, 1992; Vieira, 1997) *in* Núncio, 2001; Almeida, 1996]. In some areas with favorable ecological conditions, it is of interest that this tick is a dominant species, as in Mafra (Lisboa District, Estremadura region, mainland Portugal) and Madeira Island (Almeida, 1996; Baptista, 2006; MM Santos-Silva, personal communication). As observed in other countries, here *I. ricinus* is a permissive feeder, parasitizing a broad-range of mammalian hosts from at least six different orders, including Artiodactyla, Rodentia, Soricomorpha, Erinaceomorpha, Carnivora, and Lagomorpha (Tendeiro, 1962 *in* Dias *et al.*, 1994; Dias *et al.*, 1994; Bacellar, 1996; Caeiro, 1999; Estrada-Peña & Santos-Silva, 2005). Human parasitism by this species is also commonly reported and is associated with expected Public Health consequences (Dias *et al.*, 1994; Bacellar, 1996; Caeiro, 1999; MM Santos-Silva, personal communication). To date, seven species of tick-borne bacteria have either been isolated or detected in Portuguese *I. ricinus*, and some are already associated with human disease: *Borrelia lusitaniae*, *B. valaisiana, B. afzelii*, *B. garinii*, *B. burgdorferi s.s.*, and *R. helvetica*, beside *A. phagocytophilum* (Núncio *et al.*, 1993;

Matuschka *et al*., 1994, 1998; Bacellar *et al.*, 1999a; De Michelis *et al.*, 2000; Núncio *et al.*, 2000; Núncio, 2001; Baptista *et al.*, 2004; Baptista, 2006). It is worth mentioning that the first Portuguese isolation of *B. burgdorferi s.l.* from this tick vector reported by Núncio and coworkers (1993), was later identified as a new species, *B. lusitaniae* (Le Flèche *et al.*, 1997). The first human clinical case of LB was reported in Alentejo region by David de Morais and coworkers (1989), and this was followed by several other reports from the same area and other parts of the country (David de Morais *et al.*, 1992c, 1994; Collares-Pereira & Franca, 2000; Núncio, 2001; Baptista, 2006). The research investment in LB revealed its national importance as a tick-borne disease, and in 1999, LB was included in the list of national notifiable diseases (http://www.dgsaude.pt). Despite the pathogenic potential of several *B. burgdorferi s.l.* species that circulate in the country, so far only three species are linked to human disease, including *B. garinii*, *B. afzelii* and *B. lusitaniae*, the latter never before reported as human pathogen (Collares-Pereira *et al.*, 2004; Baptista, 2006). *R. helvetica* is another example of an *Ixodes*-borne bacterium that has recently been linked to human pathology after its detection in French patients with febrile illnesses (Fournier *et al.*, 2000) although it has not yet been identified as a cause of disease in our country. Moreover, another *Ixodes*-borne disease, HB caused by *Babesia divergens*, was reported in a splenectomized patient (Centeno-Lima *et al.*, 2003).

Given to i) the presence of *I. ricinus* in several regions of the country and its known role as a primary European vector of *A. phagocytophilum*, ii) the existence of the *A. phagocytophilum* itself in Portugal, and iii) the substantial opportunity for human exposure to the vector tick, proven by occurrence of *Ixodes*-borne diseases such as LB, the hypothesis that human *A. phagocytophilum* infections and HGA disease in Portugal were considered, further stimulating the investigation described in the following chapters. In order to promote a better awareness of tick-borne diseases, especially that caused by *A. phagocytophilum*, two revision articles were published and directed toward the practicing medical community (Appendix 1 and 2).

# CHAPTER II

## EXCHANGE TECHNOLOGY IN ANAPLASMATACEAE FIELD

## 2.1. DISTRIBUTION OF "*EHRLICHIA WALKERI*" IN *IXODES RICINUS* (ACARI: IXODIDAE) FROM THE NORTHERN PART OF ITALY*

MATSUMOTO KOUTARO, ANA SOFIA SANTOS, J STEPHEN DUMLER AND PHILIPPE BROUQUI

RESEARCH ARTICLE

JOURNAL OF MEDICAL ENTOMOLOGY 2005; 42(1):82-3

* Article published in collaboration with the Unité des Rickettsies, Faculté de Medécine, Marseille, France

SHORT COMMUNICATION

# Distribution of 'Ehrlichia walkeri' in *Ixodes ricinus* (Acari: Ixodidae) from the Northern Part of Italy

MATSUMOTO KOUTARO,[1] ANA SOPHIA SANTOS,[2] J. STEPHEN DUMLER,[3] AND PHILIPPE BROUQUI[1, 4]

J. Med. Entomol. 42(1): 82–85 (2005)

**ABSTRACT** The prevalence of 'Ehrlichia walkeri' in *Ixodes ricinus* in northern Italy was studied. A total of 456 ticks were collected from seven places around Belluno, and 292 living ticks were examined using polymerase chain reaction (PCR). Nineteen ticks were positive for *gltA* of 'E. walkeri', and the prevalence rate for each place ranged from 0 to 12.8%. The disparity of prevalence rates among areas examined suggests that 'E. walkeri' is not widespread in ticks and requires an animal host for its maintenance in nature.

**KEY WORDS** 'Ehrlichia walkeri', *gltA*, *Ixodes ricinus*, Italy

'EHRLICHIA WALKERI' IS A NEWLY detected ehrlichial species from *Ixodes ricinus* ticks for which the medical importance is yet unknown (Brouqui et al. 2003). Sequence analysis of 16S rRNA genes reveals that 'E. walkeri' is closely related to *E. ruminantium*, the agent of heartwater disease in ruminants, and is 99% related to an *Ehrlichia*-like agent described as Schotti variant (Brouqui et al. 2003). 'E. walkeri' (Schotti variant) was detected in the Netherlands (Schouls et al. 1999) and in the Baltic region of Russia (Alekseev et al. 2001), with a prevalence of 6.6 and 7.1%, respectively. In the Belluno area in Italy, it was reported to be present in 10 of 357 ticks tested (Brouqui et al. 2003). Transmission of *Ehrlichia* spp. in ticks is shown to occur transstadially but not transovarially (Groves et al. 1975, Ogden et al. 1998), suggesting that a reservoir needs to be present to maintain the organism in nature. This observation predicts that these agents are not uniformly present in all ticks but are found in areas restricted by the presence of both vector and reservoir. To confirm this hypothesis, we collected ticks in seven different biotopes located around Belluno (northern Italy) where 'E. walkeri' was originally detected and studied the prevalence of the organism in ticks using polymerase chain reaction (PCR).

## Materials and Methods

**Collection of Ticks.** Ticks were collected from seven places in and near Belluno in northern Italy, where a previous study has detected this organism (Brouqui et al. 2003) by flagging during 2 consecutive days in April 2003 (Fig. 1). Exact locations were determined by GPS values of each place. *I. ricinus* collected from three areas (sites 1–3, Losego; sites 4–6, Farra d'Alpago; site 7, Sospirolo) were used for study. Ticks were transported alive to Marseille and tested by PCR.

**DNA Extraction and PCR.** Because we expected to assess isolation of the bacteria, ticks were disinfected by immersion in 70% ethanol solution for 5 min. After two rinses with sterile water, DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Courtaboeuf, France) according to the manufacturer's manual and eluted in a final volume of 50 μl. The extracted DNA was screened by PCR specific for *gltA* of 'E. walkeri' (Inokuma et al. 2001). The primer pairs Wf319 (5′-CTAAGGTGTGCTATTTTGTC-3′) and Wr474 (5′-CTTAGCGACAAGATCATTTAC-3′) were designed based on *gltA* sequences of 'E. walkeri' (Gene Bank accession number AYO98730) (Brouqui et al. 2003) and confirmed for specificity in silico by BLASTN analysis (National Center for Biotechnology Information: http://www.ncbi.nlm.nih.gov/blast/). PCR was performed in a total volume of 25 μl of master mix consisting of 12.5 pmol of each primer, 0.5 μl of Elongase Enzyme Mix (Invitrogen, Carlsbad, CA), 20 mM of each dNTP, and 1.8 mM $MgCl_2$, and carried out under the following conditions: 94°C for 3 min, 45 cycles of 94°C for 30 s, 55°C for 30 s, 68°C for 90 s, and 68°C for 7 min. DNA from noninfected lice was used as the control of each PCR. PCR products were applied for electrophoresis on a 1% agarose gel and visualized under UV illumination after staining with ethidium bromide.

**Sequencing.** Several positive samples were randomly chosen for sequencing using the method described previously (Brouqui et al. 2003) with modifications to confirm the specificity of PCR. Briefly, PCR

[1] Unité des Rickettsies, Faculté de Médecine, 13385 Marseille, France.
[2] Centro de Estudos de Vectores e Doenças Infecciosas, Instituto Nacional de Saúde Drive Ricardo Jorge, Águas de Moura, Portugal.
[3] Department of Pathology, The Johns Hopkins Medical Institutions, Baltimore, MD 21205.
[4] E-mail: philippe.brouqui@medecine.univ-mrs.fr.

0022-2585/05/0082–0085$04.00/0 

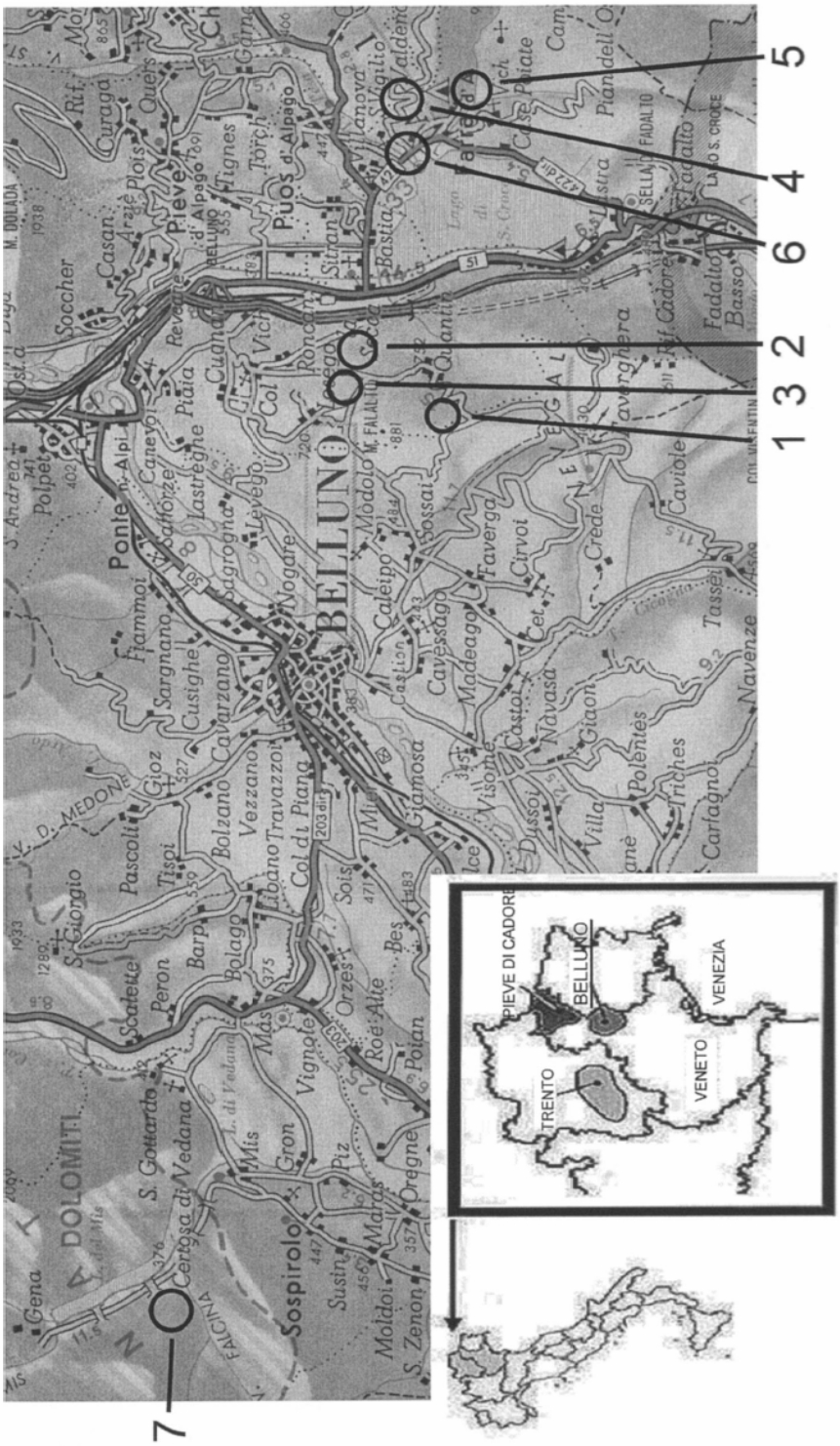



Fig. 1. Map of Belluno and suburbs in northern Italy. Numbers show the sites where ticks were collected. Sites 1–3 were in Losego, an area with wild field covered with short grasses where there was pasture and farms with sheep and goats. Sites 4–6 were in Farra d'Alpago, an area of small abandoned houses under big trees and high grass. Site 7 was in Sospirolo, a remote camping area close to a lake and distant from houses.

**Table 1. Prevalence of *E. walkeri* in seven sites around Belluno, Italy**

| Area | Biotope | Site | GPS coordinates | Stage of ticks | No. of positive/no. of samples (%) |
|---|---|---|---|---|---|
| Losego | Farms pasture, sheep and goats | 1 | N 46° 07.179′ | F | 0/5 (0.0) |
| | | | E 12° 16.132′ | M | 0/3 (0.0) |
| | | | | N | 1/20 (5.0) |
| | | | | Subtotal | 1/28 (3.6) |
| | | 2 | N 46° 08.001′ | F | 3/20 (15.0) |
| | | | E 12° 17.506′ | M | 1/12 (8.3) |
| | | | | N | 2/15 (13.3) |
| | | | | Subtotal | 6/47 (12.8) |
| | | 3 | N 46° 08.242′ | F | 1/22 (4.5) |
| | | | E 12° 17.394′ | M | 1/10 (10.0) |
| | | | | N | 4/33 (12.1) |
| | | | | Subtotal | 6/65 (9.2) |
| | | Subtotal | | | 13/140 (9.3) |
| Farra d'Alpago | High trees and grasses no farms, but abandoned houses | 4 | N 46° 07.476′ | F | 2/13 (15.4) |
| | | | E 12° 22.078′ | M | 0/8 (0.0) |
| | | | | N | 4/42 (9.5) |
| | | | | Subtotal | 6/63 (9.5) |
| | | 5 | N 46° 06.755′ | F | 0/4 (0.0) |
| | | | E 12° 22.167′ | M | 0/3 (0.0) |
| | | | | N | 0/10 (0.0) |
| | | | | Subtotal | 0/17 (0.0) |
| | | 6 | N 46° 07.496′ | F | 0/2 (0.0) |
| | | | E 12° 21.913′ | M | 0/2 (0.0) |
| | | | | N | 0/0 (N) |
| | | | | Subtotal | 0/4 (0.0) |
| | | Subtotal | | | 6/84 (7.1) |
| Sospirolo | Camping area, no house, no animal | 7[a] | N 46° 09.921′ | F | 0/9 (0.0) |
| | | | E 12° 03.533′ | M | 0/6 (0.0) |
| | | | | N | 0/53 (0.0) |
| | | | | Subtotal | 0/68 (0.0) |
| | | Total | | | 19/292 (6.5) |

N, not determined.
[a] Prevalence between site 7 and sites 2, 3, and 4 was significantly different.

products were purified using MultiScreen PCR (Millipore Corp., Bedford, MA). Primers used for PCR were also used for sequencing. PCR products were sequenced in both directions using the d-Rhodamine Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer, Coignieres, France) following the protocol of the manufacturer. Sequencing products were applied onto an automated model 3100 Applied Bio-system sequencer (Perkin-Elmer).

**Data Analysis.** Comparison of the prevalence was done using the Fisher exact test. A significant difference was set at $P < 0.05$.

## Results and Discussion

From seven places around Belluno, 456 *I. ricinus* ticks (284 nymphs, 109 adult females, and 63 adult males) were collected. Only 74% of the ticks survived. Among these, 292 ticks were examined, and 19 (6.5%) were positive for 'E. walkeri' (Table 1). Six positive samples were randomly selected and sequenced, and there was a 100% match of the 177-bp fragment with that of 'E. walkeri'. The numbers of positive ticks in each stage were 6 in 75 females (8.0%), 2 in 44 males (4.5%; adults 6.7%), and 11 in 173 nymphs (6.4%). The numbers of positive samples in each place were as follows: site 1, 1/28 (3.6%); site 2, 6/47 (12.8%); site 3, 6/65 (9.2%); site 4, 6/63 (9.5%); site 5, 0/17 (0.0%); site 6, 0/4 (0.0%); site 7, 0/68 (0.0%). The differences were significant between only site 7 and sites 2, 3, and 4 ($P < 0.01$, $<0.05$, and $<0.05$, respectively).

The average prevalence rate in this study was higher than in the previous study (2.8%, 10/357; Brouqui et al. 2003), in which ticks were collected from asymptomatic humans, although the ticks were collected from same region. This may be explained by the fact that a different PCR protocol was used, and the sensitivity in the previous study might have been lower because the primers were designed to detect all members of the genus *Ehrlichia*. This strategy yielded a 345-bp fragment, whereas the primers used in the current study were designed to specifically amplify 'E. walkeri' and yielded a smaller 177-bp fragment. An alternate explanation is that average prevalence varies with location of collection. It was expected that the frequency of 'E. walkeri' in ticks depends on location of the biotopes. In fact, the prevalence rates for each site varied between 0 and 12.8%. Of the 68 ticks collected in site 7 (Sospirolo), none were positive, whereas an average prevalence of 3.6–12.8% was found in other sites. Site 7 was the only site where no humans were living, whereas farms surrounded the other sites.

These facts suggest that 'E. walkeri' is not widely spread in ticks and that some reservoir animals are needed for maintenance. The identical prevalence rate in nymphs and adults suggests a stronger likelihood for a small mammal reservoir such as *Apodemus* or *Clethrionomys*. Trapping and animal surveillance are needed in these areas to obtain more information, particularly because reservoirs are likely to live in close contact with humans.

## Acknowledgments

This work has been supported by the European Community: EC QLK2-CT-2002–01293.

## References Cited

**Alekseev, A. N., H. V. Dubinina, Van De Pol, I., and L. M. Schouls. 2001.** Identification of *Ehrlichia* spp. and *Borrelia burgdorferi* in *Ixodes* ticks in the Baltic regions of Russia. J. Clin. Microbiol. 39: 2237–2242.

**Brouqui, P., Y. O. Sanogo, G. Caruso, F. Merola, and D. Raoult. 2003.** Candidatus *Ehrlichia walkerii*. A new *Ehrlichia* detected in *Ixodes ricinus* tick collected from asymptomatic humans in northern Italy. Ann. N. Y. Acad. Sci. 990: 134–140.

**Groves, M. G., G. L. Dennis, H. L. Amyx, D. L. Huxsoll. 1975.** Transmission of *Ehrlichia canis* to dogs by ticks (*Rhipicephalus sanguineus*). Am. J. Vet. Res. 36: 937–940.

**Inokuma, H., P. Brouqui, M. Drancourt, and D. Raoult. 2001.** Citrate synthase gene sequence: a new tool for phylogenetic analysis and identification of *Ehrlichia*. J. Clin. Microbiol. 39: 3031–3039.

**Ogden, N. H., K. Bown, B. K. Horrocks, Z. Woldehiwet, and M. Bennett. 1998.** Granulocytic *Ehrlichia* infection in ixodid ticks and mammals in woodlands and uplands of the UK Med. Vet. Entomol. 12: 423–429.

**Schouls, L. M., Van De Pol, I., S. G. Rijpkema, and C. S. Schot. 1999.** Detection and identification of *Ehrlichia*, Borrelia burgdorferi sensu lato, and *Bartonella* species in Dutch *Ixodes ricinus* ticks. J. Clin. Microbiol. 37: 2215–2222.

*Received 9 December 2003; accepted 6 February 2004.*

## 2.2. DETECTION OF A NON-PATHOGENIC VARIANT OF *ANAPLASMA PHAGOCYTOPHILUM* IN *IXODES RICINUS* FROM LA RIOJA, SPAIN*

A. PORTILLO, A.S. SANTOS, S. SANTIBÁÑEZ, L. PÉREZ-MARTÍNEZ, J.R. BLANCO, V. IBARRA, J.A. OTEO

SHORT REPORT

ANNALS OF NEW YORK ACADEMY OF SCIENCE 2005; 1063:333-6

* Article published in collaboration with the Área de Enfermedades Infecciosas, Hospitales San Millán -San Pedro – de La Rioja, Logroño, Spain

# Detection of a Non-Pathogenic Variant of *Anaplasma phagocytophilum* in *Ixodes ricinus* from La Rioja, Spain

A. PORTILLO,[a] A.S. SANTOS,[b] S. SANTIBÁÑEZ,[a] L. PÉREZ-MARTÍNEZ,[a] J.R. BLANCO,[a] V. IBARRA,[a] AND J.A. OTEO[a]

[a]*Área de Enfermedades Infecciosas, Hospitales San Millán-San Pedro-de La Rioja, Logroño, (La Rioja), Spain*

[b]*Centro de Estudos de Vectores e Doenças Infecciosas, Instituto Nacional de Saúde Dr. Ricardo Jorge, Aguas de Moura, Portugal*

ABSTRACT: **Our aim was to identify variants of *Anaplasma phagocytophilum* 16S rRNA gene sequences among products amplified from *Ixodes ricinus* collected in La Rioja, Spain. *A. phagocytophilum* AP-variant 1, reported as non-pathogenic, was detected in 12 samples (two adults and ten nymphs). This finding could justify the low incidence of human anaplasmosis in our area, despite the high prevalence of *A. phagocytophilum* in ticks.**

KEYWORDS: ***Anaplasma phagocytophilum*; human anaplasmosis; *Ixodes ricinus*; ticks; AP-Variant 1; Spain**

## INTRODUCTION

Human anaplasmosis (HA), previously designated as human granulocytic ehrlichiosis or HGE,[1] is an emerging tick-borne zoonosis transmitted by *Ixodes* ticks, specifically *Ixodes ricinus* in Europe. High prevalence of *Anaplasma* (formerly, *Ehrlichia*) *phagocytophilum* infection in *I. ricinus* from the North of Spain (La Rioja and Basque Country) has been reported.[2,3] However, to date, only sporadic cases of HA in Spain have been reported.[4,5] One hypothesis is that only a subset of the *A. phagocytophilum* strains that exist in nature cause human disease.[6–8] As little information exists about the host range and pathogenicity of different strains or variants of *A. phagocytophilum* in Europe, the objective of our study was to identify possible variants of *A. phagocytophilum* in *I. ricinus* collected in La Rioja (Spain) according to the sequence of 16S rRNA gene.

Address for correspondence: José A. Oteo, Área de Enfermedades Infecciosas. Hospitales San Millán-San Pedro-de La Rioja. Avda. Viana, No. 1. 26001, Logroño (La Rioja), Spain. Voice: 34 941 297275; fax: 34 941 297267.
jaoteo@riojasalud.es

**Ann. N.Y. Acad. Sci. 1063: 333–336 (2005).** 
**doi: 10.1196/annals.1355.053**

## MATERIAL AND METHODS

Two hundred and forty eight *I. ricinus* were tested for the pathogen. A total of 90 specimens (84 adults and 6 nymphs) were removed from cows (*Bos taurus*) in Villoslada de Cameros, a rural mountainous area from La Rioja, during November 2003. Since the collection from bovines (or any other potential animal reservoir) would favor the identification of *A. phagocytophilum* strains to which this host were susceptible and could not be representative of tick population, samples were also collected from vegetation. For this reason, in 2004 a total of 158 questing *I. ricinus* (54 adults and 104 nymphs) collected from six different sites of our region were also analyzed.

All ticks were classified according to standard taxonomic keys. Specimens were kept in 70% ethanol solution at room temperature and individually processed. Genomic DNA extraction for PCR was performed as described before.[9,10] Ticks were taken from the 70% ethanol solution, washed with sterile water, air dried and boiled for 20 min in 100 µL of 0.7 M ammonium hydroxide to free the DNA. After cooling, the vials with the lysed material were left open in a laminar airflow cabinet for 20 min at 90°C to evaporate the ammonia. The tick lysates were either used directly for PCR or stored at –20°C until used. DNA samples were pooled for initial PCR screening of 16S rRNA gene from *A. phagocytophilum* under conditions previously reported.[11] Individual samples corresponding to vials that yielded positive PCR results were additionally tested with the same primers. In addition, another pair of primers that targets the *msp2* gene was used to confirm all individual specimens that showed positive PCR results.[12] Quality control included both positive (*A. phagocytophilum* Webster, kindly provided by Dr. Dumler, from The Johns Hopkins University School of Medicine, Baltimore, USA) and negative controls that were extracted and PCR amplified in parallel with all specimens. Negative controls consisted of sterile water instead of template DNA. Subsequent sequence analysis of both strands of each amplicon of 16S rDNA [Universidad de Alcalá de Henares (Spain)] were carried out. Homology of nucleotide sequences was determined through the BLAST National Centre for Biotechnology Information (NCBI).[13]

## RESULTS

Products of the expected sizes were amplified from DNA of one of 6 nymphs removed from cows (16.6%) when tested with 16S rRNA and *msp2* primers (919 and 550 bp, respectively). *A. phagocytophilum* was not detected in adult ticks removed from cows. For ticks collected from vegetation, 9 of 104 questing nymphs (8.6%), and 2 of 54 unfed host-seeking adult ticks (3.7%) were infected with *A. phagocytophilum*. For these 11 samples, 16S rDNA and *msp2* PCR amplifications were achieved. No products were amplified from any of the negative control samples.

Sequence homology searches of 16S rRNA gene revealed that these 12 sequences analyzed were identical, and all of them differed from that of the 16S rRNA sequence of a human *A. phagocytophilum* strain (GenBank accession no. U02521) by only two nucleotides: G and A in positions 76 and 84, respectively. These changes corresponded to the AP-variant 1 strain of *A. phagocytophilum* (GenBank accession no. AY193887) previously reported as potentially non-pathogenic in USA.[6,8] These

specific nucleotide substitutions were not observed in our positive control (human-infective *A. phagocytophilum*).

## DISCUSSION

AP-variant 1 has been previously found in *I. scapularis* ticks and deer blood in the USA but never from a confirmed human infection.[14,15] Identical portions of 16S rRNA gene from European *A. phagocytophilum* are available in GenBank. Sequences that showed 100% of identity to AP-variant 1 were derived from *I. ricinus* ticks in Sweden and Germany, and from roe deer in Switzerland and Slovenia (GenBank accession nos. AJ242784; AF136713; AY281806; AF384212; AF481850, among others).[16–20] The impact of *A. phagocytophilum* 16S rRNA gene variants has been a matter of debate. The sequence variability in one gene is not sufficient to determine the genetic diversity of a certain strain. It is necessary to cultivate some of the *A. phagocytophilum* strains and to compare them in animal models in order to unravel the biological significance of the observed genetic differences. The latest advances in this field were presented at the Fourth International Conference on Rickettsiae and Rickettsial Diseases held in Logroño, Spain.[21,22]

In conclusion, AP-variant 1 strain of *A. phagocytophilum* in *I. ricinus* has been detected for the first time in Spain. To date, this variant has not been associated with human disease, and its presence may belie the few clinical cases of HA on the peninsula in contrast with the high prevalence of *A. phagocytophilum* infection found in *I. ricinus* in this region. To our knowledge, this is the first study in which *A. phagocytophilum* DNA sequences derived from ticks in Spain were compared. Further studies are needed to compare *A. phagocytophilum* DNA sequences derived from ticks and HA patients in our region.

## ACKNOWLEDGMENTS

We thank Fátima Bacellar, Rita de Sousa, and Maria Margarida Santos Silva, from the Centro de Estudos de Vectores e Doenças Infecciosas-Instituto Nacional de Saúde (Portugal), for their help and contribution to this work.

Financial support for this research was provided in part by grants from the Fondo de Investigación Sanitaria FIS (PI021810 and G03/057) from the Ministerio de Sanidad y Consumo (Spain), and also from the Fundação para a Ciência e a Tecnologia (BD/8610/2002) from the Ministério da Educação e Ensino Superior (Portugal).

[*Competing interests statement:* The authors state that they have no competing financial interests.]

## REFERENCES

1. DUMLER, J.S., A.F. BARBET, C.P.J. BEKKER, *et al.* 2001. Reorganization of genera in the families *Rickettsiaceae* and *Anaplasmataceae* in the order *Rickettsiales*: unification of some species of *Ehrlichia* with *Anaplasma*, *Cowdria* with *Ehrlichia* and *Ehrlichia*

with *Neorickettsia*, descriptions of six new species combinations and designation of *Ehrlichia equi* and "HGE agent" as subjective synonyms of *Ehrlichia phagocytophila*. Int. J. Syst. Evol. Microbiol. **51:** 2145–2165.
2. OTEO, J.A., H. GIL, M. BARRAL, *et al.* 2001. Presence of granulocytic *Ehrlichia* in ticks and serological evidence of human infection in La Rioja, Spain. Epidemiol. Infec. **127:** 353–358.
3. BARRAL, M., L. BENEDICTO, H. GIL, *et al.* 1999. Detection of *Ehrlichia phagocytophila* DNA in *Ixodes ricinus* from areas in the North of Spain. *In* Rickettsiae and Rickettsial Diseases at the Turn of the Third Millennium. D. Raoult & P. Brouqui, Eds.: 428–431. Elsevier. Paris.
4. OTEO, J.A., J.R. BLANCO, V. MARTÍNEZ DE ARTOLA, *et al.* 2000. First report of human granulocytic ehrlichiosis from Southern Europe (Spain). Emerg. Infect. Dis. **6:** 430–431.
5. GARCÍA, J.C., M.J. NÚÑEZ, B. CASTRO, *et al.* 2006. Human granulocytic anaplasmosis: the first Spanish case confirmed by PCR. Ann. N.Y. Acad. Sci. In press.
6. MASSUNG, R.F., M.J. MAUEL, J.H. OWENS, *et al.* 2002. Genetic variants of *Ehrlichia phagocytophila*, Rhode Island and Connecticut. Emerg. Infect. Dis. **8:** 467–472.
7. STUEN, S., I. VAN DE POL, K. BERGSTRÖM, *et al.* 2002. Identification of *Anaplasma phagocytophila* (formerly *Ehrlichia phagocytophila*) variants in blood from sheep in Norway. J. Clin. Microbiol. **40:** 3192–3197.
8. MASSUNG, R.F., R.A. PRIESTLEY, N.J. MILLER, *et al.* 2003. Inability of a variant strain of *Anaplasma phagocytophilum* to infect mice. J. Infect. Dis. **188:** 1757–1763.
9. GUY, E.C. & G. STANEK. 1991. Detection of *Borrelia burgdorferi* in patients with Lyme disease by the polymerase chain reaction. J. Clin. Pathol. **44:** 610–611.
10. RIJPKEMA, S., D. GOLUBIC, M. MOLKENBOER, *et al.* 1996. Identification of four genomic groups of *Borrelia burgdorferi* sensu lato in *Ixodes ricinus* ticks collected in Lyme borreliosis endemic region of northern Croatia. Exp. Appl. Acarol. **20:** 23–30.
11. CHEN, S.M., J.S. DUMLER, J.S. BAKKEN, *et al.* 1994. Identification of granulocytotropic *Ehrlichia* species as the etiologic agent of human disease. J. Clin. Microbiol. **32:** 589–595.
12. CASPERSEN, K., J.H. PARK, S. PATIL, *et al.* 2002. Genetic variability and stability of *Anaplasma phagocytophila msp2* (p44). Infect. Immun. **70:** 1230–1234.
13. http://www.ncbi.nlm.gov/BLAST/
14. BELONGIA, E.A., K.D. REED, P.D. MITCHELL, *et al.* 1997. Prevalence of granulocytic *Ehrlichia* infection among white-tailed deer in Wisconsin. J. Clin. Microbiol. **35:** 1465–1468.
15. MASSUNG, R.F., M.J. MAUEL, J.H. OWENS, *et al.* 1998. Nested PCR assay for detection of granulocytic ehrlichiae. J. Clin. Microbiol. **36:** 1090–1095.
16. VON STEDINGK, L.V., M. GURTELSCHMID, H.S. HANSON, *et al.* 1997. The human granulocytic ehrlichiosis (HGE) agent in Swedish ticks. Clin. Microbiol. Infect. **3:** 573–574.
17. BAUMGARTEN, B.U., M. ROLLINGHOFF & C. BOGDAN. 1999. Prevalence of *Borrelia burgdorferi* and granulocytic and monocytic ehrlichiae in *Ixodes ricinus* ticks from Southern Germany. J. Clin. Microbiol. **37:** 3448–3451.
18. VON LOEWENICH, F.D., B.U. BAUMGARTEN, K. SCHRÖPPEL, *et al.* 2003. High diversity of *ankA* sequences of *Anaplasma phagocytophilum* among *Ixodes ricinus* ticks in Germany. J. Clin. Microbiol. **41:** 5033–5040.
19. LIZ, J.S., J.W. SUMNER, K. PFISTER, *et al.* 2002. PCR detection and serological evidence of granulocytic ehrlichial infection in roe deer (*Capreolus capreolus*) and chamois (*Rupicapra rupicapra*). J. Clin. Microbiol. **40:** 892–897.
20. PETROVEC, M., A. BIDOVEC, J.W. SUMNER, *et al.* 2002. Infection with *Anaplasma phagocytophila* in cervids from Slovenia: evidence of two genotypic lineages. Wien. Klin. Wochenschr. **114:** 641–647.
21. MASSUNG, R.F., M.L. LEVIN, N.J. MILLER, *et al.* 2005. Reservoir competency of goats for *Anaplasma phagocytophilum*. Presented at the Fourth International Conference on Rickettsiae and Rickettsial Diseases. Logroño, Spain, June 18.
22. MASSUNG, R.F., M.L. LEVIN, U.G. MUNDERLOH, *et al.* 2005. Isolation of *Anaplasma phagocytophilum* strain AP-variant 1 in a tick-derived cell line. Presented at the Fourth International Conference on Rickettsiae and Rickettsial Diseases. Logroño, Spain, June 18.

# CHAPTER III

## *ANAPLASMA PHAGOCYTOPHILUM* IN TICKS (ACARI:IXODIDAE)

# 3.1. DETECTION OF *ANAPLASMA PHAGOCYTOPHILUM* DNA IN *IXODES* TICKS (ACARI:IXODIDAE) FROM MADEIRA ISLAND AND SETÚBAL DISTRICT, MAINLAND PORTUGAL

ANA SOFIA SANTOS, MARGARIDA SANTOS-SILVA, VICTOR CARLOS ALMEIDA, FÁTIMA BACELLAR, AND JOHN STEPHEN DUMLER

RESEARCH ARTICLE

EMERGING INFECTIOUS DISEASES 2004; 10(9):1643-8

# Detection of *Anaplasma phagocytophilum* DNA in *Ixodes* Ticks (Acari: *Ixodidae*) from Madeira Island and Setúbal District, Mainland Portugal

**Ana Sofia Santos,* Maria Margarida Santos-Silva,* Victor Carlos Almeida,† Fátima Bacellar,* and John Stephen Dumler‡**

A total of 278 *Ixodes* ticks, collected from Madeira Island and Setúbal District, mainland Portugal, were examined by polymerase chain reaction (PCR) for the presence of *Anaplasma phagocytophilum*. Six (4%) of 142 *Ixodes ricinus* nymphs collected in Madeira Island and 1 nymph and 1 male (2%) of 93 *I. ventalloi* collected in Setúbal District tested positive for *A. phagocytophilum msp*2 genes or *rrs*. Infection was not detected among 43 *I. ricinus* on mainland Portugal. All PCR products were confirmed by nucleotide sequencing to be identical or to be most closely related to *A. phagocytophilum*. To our knowledge, this is the first evidence of *A. phagocytophilum* in ticks from Setúbal District, mainland Portugal, and the first documentation of *Anaplasma* infection in *I. ventalloi*. Moreover, these findings confirm the persistence of *A. phagocytophilum* in Madeira Island's *I. ricinus*.

*Anaplasma phagocytophilum* (formerly *Ehrlichia phagocytophila*, *E. equi*, and the human granulocytic ehrlichiosis agent [HGE agent] [1]) is well established as a worldwide tickborne agent of veterinary importance and is considered an emerging human pathogen. The initial reports of human disease caused by *A. phagocytophilum*, now called human granulocytic anaplasmosis, came from Minnesota and Wisconsin in 1994 (2,3). Human granulocytic anaplasmosis is an acute, nonspecific febrile illness characterized by headache, myalgias, malaise, and hematologic abnormalities, such as thrombocytopenia and leukopenia as well as elevated levels of hepatic transaminases (4). Since that first report, an increasing number of cases have been described, mostly in the upper Midwest and in the Northeast regions of the United States (5). Three years later, in 1997, acute cases of this disease were also described in Europe (6,7). Several serologic and polymerase chain reaction (PCR)-based studies described the wide distribution of *A. phagocytophilum* across Europe and in some parts of the Middle East and Asia (8–10). Nevertheless, confirmed cases of human granulocytic anaplasmosis are rare; most European cases are described in Slovenia (11), with only a few reports from other European countries (12) and China (13).

The ecology of *A. phagocytophilum* is still being defined, but the agent is thought to be maintained in nature in a tick-rodent cycle, similar to that of *Borrelia burdgdorferi* (the agent of Lyme disease), with humans being involved only as incidental "dead-end" hosts (14–17). Exposure to tick bites is considered to be the most common route of human infection, although human granulocytic anaplasmosis has been reported after perinatal transmission or contact with infected animal blood (18,19). *A. phagocytophilum* is associated with *Ixodes* ticks that are known vectors, including *I. scapularis*, *I. pacificus*, and *I. spinipalpis* in the United States (15,20,21), *I. ricinus* mostly in southern, central and northern European regions (22–26), *I. trianguliceps* in the United Kingdom (27), and *Ixodes persulcatus* in eastern parts of Europe (28) and Asia (9).

In Portugal little information is available concerning the epidemiology of *A. phagocytophilum*; the agent was documented only once in *I. ricinus* ticks from Madeira Island (Núncio MS, et al, unpub data). However, the true prevalence and public health impact of *A. phagocytophilum*

*Instituto Nacional de Saúde Dr. Ricardo Jorge, Águas de Moura, Portugal; †Direcção Regional de Pecuária, Funchal, Portugal; and ‡Johns Hopkins University School of Medicine, Baltimore, Maryland, USA

is likely underestimated since little research has been conducted on this bacterium in Portugal. In fact, seasonal outbreaks of enzootic abortions and unspecific febrile illness (commonly named pasture fever) in domestic ruminants, which could be attributable to *A. phagocytophilum*, have been known to breeders and veterinarians across the country for years. Thus, to expand knowledge of *A. phagocytophilum* in Portugal, a detailed investigation was initiated. The preliminary results concerning agent distribution are presented here. The purpose of this study was to investigate both the persistence of *A. phagocytophilum* on Madeira Island, where it was initially described, and the presence of the agent in *Ixodes* ticks from mainland Portugal.

## Materials and Methods

### Tick Sampling

During 2003 and the beginning of 2004, adults and nymphs were collected from one site on Madeira Island (site 1, Paúl da Serra–Porto Moniz) and from five different sites in the Setúbal District, mainland Portugal (site 2, Barris–Palmela; site 3, Baixa de Palmela; site 4, Picheleiros–Azeitão, site 5, Azeitão, site 6, Maçã–Sesimbra) (Figure 1). Most ticks were unfed, actively questing arthropods; they were obtained by flagging vegetation on pastures and wooded areas bordering farms and country houses. In site 3, additional specimens were also collected from domestic cats (*Felis catus domesticus*). The ticks were identified by morphologic characteristics according to standard taxonomic keys (29,30).

### Preparation of DNA Extracts from Ticks

Ticks were processed individually as described (25). Briefly, each tick was taken from the 70% ethanol solution used for storage, air dried, and boiled for 20 min in 100 µL of 0.7 mol/L ammonium hydroxide to free DNA. After cooling, the vial with the lysate was left open for 20 min at 90°C to evaporate the ammonia. The tick lysate was used directly for PCR. To monitor for occurrence of false-positive samples, negative controls were included during extraction of the tick DNA (one control sample for each six tick samples, with a minimum of two controls).

### PCR Amplification

DNA amplifications were performed in a Biometra T-3 thermoblock thermal cycler (Biometra GmbH, Göttingen, Germany) with two sets of primers: msp465f and msp980r, derived from the highly conserved regions of major surface protein-2 (*msp2*) paralogous genes of *A. phagocytophilum* (31), and ge9f and ge10r, which amplify a fragment of the 16S rRNA gene of *A. phagocytophilum* (3). PCR was performed in a total volume of 50 µL that contained 1 µmol/L of each primer, 2.5 U of Taq DNA polymerase (Roche, Mannheim, Germany), 200 µmol/L of each deoxynucleotide triphosphate (GeneAmp PCR Reagent Kit, Perkin-Elmer, Foster City, CA), 10 mmol/L Tris HCL, 1.5 mmol/L $MgCl_2$, and 50 mmol/L KCl pH 8.3 (Roche), as described (3,31). Adult ticks were tested individually by using 5 µL of DNA extract. Nymphs were pooled according to geographic site, up to a maximum of 10 different tick extracts per reaction, and 10 µL of the pooled DNA was used for initial screening. All positive pools were confirmed in a second PCR round that used 5 µL of original DNA extract from each nymph. PCR products were separated on 1.5% agarose by electrophorectic migration, stained with ethidium bromide, and visualized under UV light. Quality controls included both positive and negative controls that were PCR amplified in parallel with all specimens. To minimize contamination, DNA preparation with setup, PCR, and sample analysis were performed in three separate rooms.

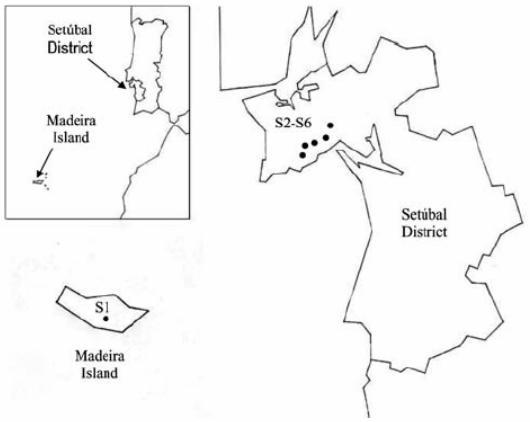



Figure 1. Collection sites in Madeira Island and Setúbal District, mainland Portugal. S, collection site.

### DNA Sequencing and Data Analysis

Each positive PCR product was sequenced after DNA purification by a MiniElute PCR Purification Kit (Qiagen, Valencia, CA). For DNA sequencing, the BigDye terminator cycle sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA), was used as recommended by the manufacturer. Sample amplifications were performed with the forward and reverse primers used for PCR identification (3,31), with the following modifications: 25 cycles of 96°C for 10 s, 4°C below the melting temperature of each primer for 5 s, and 60°C for 4 min. Dye Ex 96 Kit (Qiagen) was used to remove the dye terminators. Sequences were determined with a 3100 Genetic Analyzer sequencer (Applied Biosystems). After review and editing, sequence homology searches were made by BLASTN

analysis of GenBank. Sequences were aligned by using ClustalX (32) with the neighbor-joining protocol and 1,000 bootstrap replications, and comparing with the 2 *msp2* paralogs of *A. phagocytophilum* Webster strain (AY253530 and AF443404), one *msp2* paralog of USG3 strain (AF029323), and with *A. marginale msp2* (AY138955) and *msp3* (AY127893) as outgroups. Dendrograms illustrating the similarity of *msp2*s were visualized with TreeView (33).

## Results

A total of 278 *Ixodes* ticks were tested for *A. phagocytophilum* DNA, including 142 *I. ricinus* from Madeira Island and 43 *I. ricinus* and 93 *I. ventalloi* from Setúbal District. The site of collection, origin, and tick stage are shown in Table 1 and Figure 1. PCR performed with the *msp2* primers detected *A. phagocytophilum* DNA in seven pools of nymphs (six pools of 10 *I. ricinus* from site 1, Madeira Island, and one pool of 4 *I. ventalloi* from site 3, Setúbal District) and also in 1 male *I. ventalloi* from site 3, Setúbal District, as demonstrated by the characteristic 550-bp band. PCRs conducted on individual ticks that comprised positive pools confirmed the results and showed that only one nymph per positive pool contained *A. phagocytophilum* DNA (Tables 1 and 2). PCR test results were negative for all *I. ricinus* collected in the sites in Setúbal District. Overall, the infection rate was 6 (4%) of 142 for *I. ricinus* and 2 (2%) of 93 for *I. ventalloi*. Analysis based on direct amplicon sequencing showed the expected conserved 5′ end followed by ambiguous sequences that corresponded to the hypervariable central region of *msp2*, as anticipated based on the presence of >52 *msp2* copies in the *A. phagocytophilum* HZ strain genome (34). Thus, for appropriate comparison and alignment, the *msp2* 5′ sequences were edited from the positions where unambiguous reads could be determined and terminated 70 nt into the sequence at the approximate beginning of the hypervariable region. A similar alignment protocol for the 3′ end of the *msp2* amplicons showed more ambiguous positions, which prohibited effective alignment and sequence determination. Thus, *msp2* sequence alignments depended upon approximately 70 nt 5′ to the hypervariable region and were performed less for phylogenetic stratification of *A. phagocytophilum* in the ticks than to confirm that the amplified *msp2* sequences were not derived from other related *Anaplasma* or *Ehrlichia* spp. The nucleotide sequences determined for this 70-bp region amplified from all eight ticks showed 98.5%–85.7% similarity, 94.2%–86.9% similarity when compared to representative *msp2* sequences of *A. phagocytophilum* Webster and USG3 strains, and 63.7%–35.0% similarity when compared to *A. marginale msp2* and *msp3* sequences (Figure 2). Sequences obtained from the two *I. ventalloi* from mainland Portugal clustered together and separately from other *msp2* sequences obtained from *I. ricinus* on Madeira Island (Figure 2).

When amplified by using *rrs* primers ge9f and ge10r, compared to *A. phagocytophilum* U02521, sequences were 99% identical to two *I. ventalloi* (636/640 positions and 846/848 positions, respectively) on mainland Portugal and to three *I. ricinus* (836/841, 817/820, and 838/839 positions, respectively) on Madeira Island.

## Discussion

This study constitutes part of a larger effort to investigate the distribution of *A. phagocytophilum* in various regions of Portugal. Our data provide supporting evidence that *A. phagocytophilum* is present in actively questing *I. ricinus* from Madeira Island and in *I. ventalloi* from Setúbal District, mainland Portugal.

We used two approaches for identifying *A. phagocytophilum* in ticks: 1) standard amplification of *rrs* that can have limited sensitivity because of a single copy in each bacterial genome, and 2) amplification of *msp2*, a gene for which as many as 52 paralogs are present in the *A. phagocytophilum* genome and for which detection sensitivity is

Table 1. Results of PCR to detect *Anaplasma phagocytophilum* DNA in ticks[a]

| Area | Site | Origin | *Ixodes ricinus* | | | *I. ventalloi* | | | Total[c] |
|---|---|---|---|---|---|---|---|---|---|
| | | | Nymphs[b] | F[b] | M[b] | Nymphs[b] | F[b] | M[b] | |
| Madeira Island | | | | | | | | | |
| Paúl da Serra–Porto Moniz | 1 | Vegetation | 6/139 | 0/2 | 0/1 | – | – | – | 142 |
| Setúbal District Portugal Mainland | | | | | | | | | |
| Barris–Palmela | 2 | Vegetation | 0/1 | 0/5 | 0/7 | – | – | 0/1 | 14 |
| Baixa de Palmela | 3 | Vegetation | 0/2 | 0/2 | 0/2 | 1/15 | 0/6 | 0/7 | 34 |
| | | *Felis catus domesticus* | – | – | – | – | 0/6 | 1/4 | 10 |
| Picheleiros–Azeitão | 4 | Vegetation | – | 0/2 | 0/2 | 0/12 | 0/9 | 0/18 | 43 |
| Azeitão | 5 | Vegetation | – | – | 0/1 | – | – | 0/1 | 2 |
| Maçã–Sesimbra | 6 | Vegetation | – | 0/10 | 0/9 | 0/1 | 0/4 | 0/9 | 33 |
| Total[c] | | | 142 | 21 | 22 | 28 | 25 | 40 | 278 |

[a]PCR, polymerase chain reaction; F, female; M, male.
[b]Number of positives ticks/number of ticks examined.
[c]Total number of ticks examined.

Table 2. PCR-positive results of ticks[a]

| Sites | No. positive nymphs | No. positive adults | Tick extracts codes |
|---|---|---|---|
| Madeira Island | | | |
| 1 | 6 | – | 11; 60; 93; 118; 122; 137 |
| Setúbal District Mainland Portugal | | | |
| 3 | 1 | 1 | 160; 246 (respectively) |

[a]PCR, polymerase chain reaction.

enhanced (34). The pitfall of *msp2* amplification derives from targeting conserved sequences that flank a hypervariable central region, which results in amplicons with partial sequence ambiguity when cloning is not attempted before sequencing (31). These findings are highly unlikely to represent amplicon contamination since marked sequence diversity was observed, and since only a single tick from each pool was positive in each reaction. Although only limited data can gleaned by this analysis, which interrogates only nucleic acids of small size, Casey et al. have shown that *msp2* "similarity" groups, reflecting clusters determined by a similar sequencing approach, can be useful in predicting phylogenetic relationships, particularly with reference to adaptation to specific host niches (35).

Madeira, the main island of the Madeira Archipelago, is located in the North Atlantic Ocean, about 800 km west of the African continent and 1,000 km from the European coast. On this island, *I. ricinus* is the most abundant tick species and the only *Ixodes* tick that was found in this study. *A. phagocytophilum* was detected in 4% of *I. ricinus* collected in Paúl da Serra. Our results corroborate previous findings, although prevalence here is slightly lower than the 7.5% infection rate in ticks previously collected in similar areas (Núncio MS, et al., unpub data). These differences may be attributable to seasonal variations in *A. phagocytophilum* prevalence within reservoir hosts or ticks or to technical aspects of detection. Regardless, studies that use a greater number of samples and that are performed in different seasons, locations, and habitats will be needed to confirm the levels of infection. Nevertheless, these findings are generally similar to those described elsewhere in Europe, although prevalence rates can vary greatly with the origin of *I. ricinus* examined, ranging from a minimum of ≤1% in the United Kingdom, France, and Sweden (23,24,36) to a maximum of 24% to 29% in northern Italy, Germany, and Spain (22,25,26). The public health importance of these findings still remains to be determined. *I. ricinus* is an exophilic, three-host tick known to bite several domestic animals and humans in Portugal (30). Therefore, we can assume that the presence of *A. phagocytophilum* on Madeira Island *I. ricinus* suggests a potential health threat to animals and humans and should be investigated.

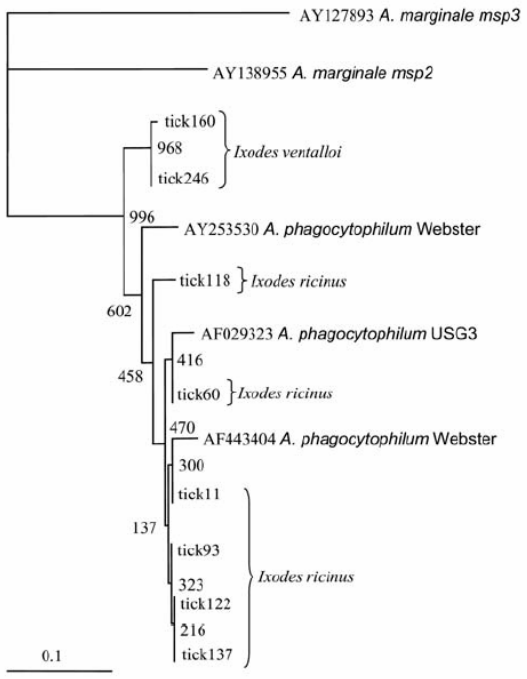



Figure 2. Dendrogram showing the phylogenetic relationships of the msp2 sequences of the newly identified strains and other representative sequences from North American *Anaplasma phagocytophilum* strains (Webster strain–Wisconsin and USG3 strain–eastern United States), and from *A. marginale* Florida strain (*msp2* and *msp3*). Bootstrap values (out of 1,000 iterations) are shown at the nodes. Bar, substitutions/1,000 bp.

Mainland Portugal is the most western region of Europe, with an area of 89,000 km$^2$, divided into 18 districts. Although *I. ricinus* is not the main tick species in mainland Portugal, it can be found across the country in habitats with favorable conditions. Focused in Setúbal District, to the south of the Tejo River, our study detected *I. ricinus* in all five sites chosen for field work: Barris; Baixa de Palmela; Picheleiros; Azeitão, and Maçã. In those sites, the distribution of *I. ricinus* was accompanied by another *Ixodes* species, *I. ventalloi*. Another ecologically interesting finding that should be further confirmed was that, although all of the *I. ricinus* from mainland Portugal tested negative, evidence of *A. phagocytophilum* was found in 2% of all *I. ventalloi,* including 5% collected in Baixa de Palmela. The *msp2* sequences identified in these two ticks were more closely related to each other than to any *msp2* sequence identified in ticks from Madeira Island. In contrast, *A. phagocytophilum msp2* diversity in *I. ricinus* from Madeira Island was broad and showed overlap with gene sequences identified in North American strains,

as observed for some *A. phagocytophilum* strains in the United Kingdom (35).

To our knowledge, this identification of *A. phagocytophilum* in ticks is the first from mainland Portugal and the first documentation of *Anaplasma* infection in *I. ventalloi*. This species is an endophilic, three-host tick well adapted to a broad range of habitats that vary from open, dry forest in semidesert Mediterranean areas to the mild humid conditions in the southern part of the British Isles. In Portugal, *I. ventalloi* infest a variety of small rodents, carnivores, and lizards but have not been found to feed on humans (30). *A. phagocytophilum* has already been reported in other ticks, besides the known vector species (37–41). The presence in alternate ticks is attributable to the existence of secondary maintenance cycles, in which *A. phagocytophilum* circulates between relatively host-specific, usually nonhuman-biting ticks and their hosts (38,39). Those additional cycles would buffer the agent from local extinction and help reestablish the primary cycles (38,39). Although this hypothesis might explain our results, the competency of *I. ventalloi* to act as vector for *A. phagocytophilum* has yet to be demonstrated. Moreover, the different average prevalences observed in each location suggest that *A. phagocytophilum* is not widely spread in ticks and that some reservoir animals or hosts are needed for its maintenance. Trapping and animal surveillance are needed to provide more information that could help to explain the biological importance of those findings.

### Acknowledgments

We thank Maria Arminda Santos for useful help in field work and Sonia Pedro from National Health Insitute Ricardo Jorge for valuable assistance with sequencing.

This research was partially supported by the Portuguese government through the Fundação para a Ciência e a Tecnologia grant BD/8610/2002 and through U.S. National Institutes of Health grant R01-AI41213 to J.S.D.

Ms. Santos works at the Centre of Vector-Borne and Infectious Diseases, National Institute of Health, Portugal. Her research has been focused on tickborne agents, mainly rickettsial agents with human health importance. She is pursuing a doctoral degree focused on human anaplasmosis and ehrlichiosis in Portugal.

### References

1. Dumler JS, Barbet AF, Bekker CP, Dasch GA, Palmer GH, Ray SC, et al. Reorganization of genera in the families *Rickettsiaceae* and *Anaplasmataceae* in the order Rickettsiales: unification of some species of *Ehrlichia* with *Anaplasma*, *Cowdria* with *Ehrlichia* and *Ehrlichia* with *Neorickettsia*, descriptions of six new species combinations and designation of *Ehrlichia equi* and "HGE agent" as subjective synonyms of *Ehrlichia phagocytophila*. Int J Syst Evol Microbiol. 2001;51:2145–65.
2. Bakken JS, Dumler JS, Chen S-M, Eckman MR, Van Etta LL, Walker DH. Human granulocytic ehrlichiosis in the upper midwest United States, a new species emerging? JAMA. 1994;272:212–8.
3. Chen S-M, Dumler JS, Bakken JS, Walker DH. Identification of a granulocytotropic *Ehrlichia* species as the etiologic agent of human diseases. J Clin Microbiol. 1994;32:589–95.
4. Bakken JS, Krueth J, Wilson-Nordskog C, Tilden RL, Asanovich K, Dumler JS. Clinical and laboratory characteristics of human granulocytic ehrlichiosis. JAMA. 1996;275:199–205.
5. Bakken JS, Dumler JS. Human granulocytic ehrlichiosis. Clin Infect Dis. 2000;31:554–60.
6. Petrovec M, Furlan SL, Zupanc TA, Strle F, Brouqui P, Roux V, et al. Human disease in Europe caused by a granulocytic *Ehrlichia* species. J Clin Microbiol. 1997;35:1556–9.
7. Lotric-Furlan S, Petrovec M, Zupanac TA, Nicholson WL, Sumner JW, Childs JE, et al. Human granulocytic ehrlichiosis in Europe: clinical and laboratory findings for four patients from Slovenia. Clin Infect Dis. 1998;27:424–8.
8. Keysary A, Amram L, Keren G, Sthoeger Z, Potasman I, Jacob A, et al. Serologic evidence of human monocytic and granulocytic ehrlichiosis in Israel. Emerg Infect Dis. 1999;5:775–8.
9. Cao WC, Zhao QM, Zhang PH, Dumler JS, Zhang XT, Fang LQ, et al. Granulocytic Ehrlichiae in *Ixodes persulcatus* ticks from an area in China where Lyme disease is endemic. J Clin Microbiol. 2000;38:4208–10.
10. Park JH, Heo EJ, Choi KS, Dumler JS, Chae JS. Detection of antibodies to *Anaplasma phagocytophilum* and *Ehrlichia chaffeensis* antigens in sera of Korean patients by western immunoblotting and indirect immunofluorescence assays. Clin Diagn Lab Immunol. 2003;10:1059–64.
11. Lotric-Furlan S, Petrovec M, Avsic-Zupanc T, Strle F. Human granulocytic ehrlichiosis in Slovenia. Ann N Y Acad Sci. 2003;990:279–84.
12. Blanco JR, Oteo JA. Human granulocytic ehrlichiosis in Europe. Clin Microbiol Infect. 2002;8:763–72.
13. Gao D, Cao W, Zhang X. Investigations on human ehrlichia infectious people in Daxingan Mountains [article in Chinese]. Zhonghua Liu Xing Bing Xue Za Zhi. 2001;22:137–41.
14. Lane RS, Piesman J, Burgdorfer W. Lyme borreliosis: relation of its causative agent to its vector s and hosts in North America and Europe. Annu Rev Entomol. 1991;36:587–609.
15. Pancholi P, Kolbert CP, Mitchell PD, Reed KD Jr, Dumler JS, Bakken JS, et al. *Ixodes dammini* as a potential vector of human granulocytic ehrlichiosis. J Infect Dis. 1995;172:1007–12.
16. Daniels TJ, Falco RC, Schwartz I, Varde S, Robbins RG. Deer ticks (*Ixodes scapularis*) and the agents of Lyme disease and human granulocytic ehrlichiosis in a New York City park. Emerg Infect Dis. 1997;3:353–5.
17. Levin LM, des Vignes F, Fish D. Disparity in the natural cycles of *Borrelia burgdorferi* and the agent of human granulocytic ehrlichiosis. Emerg Infect Dis. 1999;5:204–8.
18. Bakken JS, Krueth J, Lund T, Malkovitch D, Asanovich K, Dumler JS. Exposure to deer blood may be a cause of human granulocytic ehrlichiosis. Clin Infect Dis. 1996;23:198.
19. Horowitz HW, Kilchevsky E, Haber S, Aguero-Rosenfeld M, Kranwinkel R, James EK, et al. Perinatal transmission of the agent of human granulocytic ehrlichiosis. N Engl J Med. 1998;339:375–8.
20. Richter PJ, Kimsey RB, Madigan JE, Barlough JE, Dumler JS, Brooks DL. *Ixodes pacificus* (Acari: Ixodidae) as a vector of *Ehrlichia equi* (Rickettsiales: Ehrlichieae). J Med Entomol. 1996;33:1–5.
21. Burkot TR, Maupin GO, Schneider BS, Denatale C, Happ CM, Rutherford JS, et al. Use of a sentinel host system to study the questing behavior of *Ixodes spinipalpis* and its role in the transmission of *Borrelia bissettii*, human granulocytic ehrlichiosis, and *Babesia microti*. Am J Trop Med Hyg. 2001;65:293–9.

22. Cinco M, Padovan D, Murgia R, Maroli M, Frusteri L, Heldtander M, et al. Coexistence of *Ehrlichia phagocytophila* and *Borrelia burgdorferi sensu lato* in *Ixodes ricinus* ticks from Italy as determined by 16S rRNA gene sequencing. J Clin Microbiol. 1997;35:3365–6.
23. Guy E, Tasker S, Joynson DH. Detection of the agent of human granulocytic ehrlichiosis (HGE) in UK ticks using polymerase chain reaction. Epidemiol Infect. 1998;121:681–3.
24. Pusterla N, Leutenegger CM, Huder JB, Weber R, Braun U, Lutz H. Evidence of the human granulocytic ehrlichiosis agent in *Ixodes ricinus* ticks in Switzerland. J Clin Microbiol. 1999;37:1332–4.
25. Schouls LM, Van de Pol I, Rijpkema SGT, Schot C S. Detection and identification of *Ehrlichia*, *Borrelia burgdorferi* sensu lato and *Bartonella* species in Dutch *Ixodes ricinus* ticks. J Clin. Microbiol. 1999;37:2215–22.
26. Oteo JA, Gil H, Barral, M, Pérez A, Jimenez S, Blanco JR, et al. Presence of granulocytic ehrlichia in ticks and serological evidence of human infection in La Rioja, Spain. Epidemiol Infect. 2001;127:353–8.
27. Ogden NH, Bown K, Horrocks BK, Woldehiwet Z, Bennett M. Granulocytic *Ehrlichia* infection in ixodid ticks and mammals in woodlands and uplands of the U.K. Med Vet Entomol. 1998;12:423–9.
28. Alekseev AN, Dubinina HV, Van De Pol I, Schouls LM. Identification of *Ehrlichia spp*. and *Borrelia burgdorferi* in *Ixodes* ticks in the Baltic regions of Russia. J Clin Microbiol. 2001;39:2237–42.
29. Cordas T, Aeschlimann A, Morel PC. Étude morphologique des ixodidae s. str. (Schulze, 1937) de Suisse au microscope électronique à balayage. Acaralogia. 1993;34:21–46.
30. Dias JA. As carraças (Acari-Ixodoidea) da Península Ibérica. Algumas considerações sobre a sua biogeografia e relacionamento com a ixodofauna afropaleártica e afrotropical. Estudos, Ensaios e Documentos. 1994;158:1–163.
31. Caspersen K, Park J-H, Patil S, Dumler JS. Genetic variability and stability of *Anaplasma phagocytophila* msp2 (p44). Infect Immun. 2002;70:1230–4.
32. Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 1997;24:4876–82.
33. Page RD. TreeView: an application to display phylogenetic trees on personal computers. Comput Appl Biosci. 1996;12:357–8.
34. Scorpio DG, Caspersen K, Ogata H, Park J, Dumler JS. Restricted changes in major surface protein-2 (*msp2*) transcription after prolonged in vitro passage of *Anaplasma phagocytophilum*. BioMedCentral Microbiology. 2004;4:1.
35. Casey AN, Birtles RJ, Radford AD, Bown KJ, French NP, Woldehiwet Z, et al. Groupings of highly similar major surface protein (*p44*)-encoding paralogues: a potential index of genetic diversity amongst isolates of *Anaplasma phagocytophilum*. Microbiology. 2004;150:727–34.
36. Parola P, Beati L, Cambon M, Brouqui P, Raoult D. Ehrlichial DNA amplified from *Ixodes ricinus* (Acari: Ixodidae) in France. J Med Entomol. 1998;35:180–3.
37. Zeidner NS, Burkot TR, Massung R, Nicholson WL, Dolan MC, Rutherford JS, et al. Transmission of the agent of human granulocytic ehrlichiosis by *Ixodes spinipalpis* ticks: evidence of an enzootic cycle of dual infection with *Borrelia burgdorferi* in northern Colorado. J Infect Dis. 2000;12:616–9.
38. Bown KJ, Begon M, Bennett M, Woldehiwet Z, Ogden NH. Seasonal dynamics of *Anaplasma phagocytophila* in a rodent-tick (*Ixodes trianguliceps*) system, United Kingdom. Emerg Infect Dis. 2003;9:63–70.
39. Goethert HK, Telford SR 3rd. Enzootic transmission of the agent of human granulocytic ehrlichiosis among cottontail rabbits. Am J Trop Med Hyg. 2003;68:633–7.
40. Holden K, Boothby JT, Anand S, Massung RF. Detection of *Borrelia burgdorferi*, *Ehrlichia chaffeensis*, and *Anaplasma phagocytophilum* in ticks (Acari: Ixodidae) from a coastal region of California. J Med Entomol. 2003;40:534–9.
41. Sixl W, Petrovec M, Marth E, Wust G, Stunzner D, Schweiger R, et al. Investigation of *Anaplasma phagocytophila* infections in *Ixodes ricinus* and *Dermacentor reticulatus* ticks in Austria. Ann N Y Acad Sci. 2003;990:94–7.

Address for correspondence: Ana Sofia Santos, Centro de Estudos de Vectores e Doenças Infecciosas, Instituto Nacional de Saúde Dr. Ricardo Jorge, Avenida da Liberdade 5, 2965-575 Águas de Moura, Portugal; fax: +351-265-912568; email: ana.santos@insa.min-saude.pt

## 3.2. TICKS PARASITIZING WILD BIRDS IN PORTUGAL: DETECTION OF *RICKETTSIA AESCHLIMANNII*, *R. HELVETICA* AND *R. MASSILIAE*

MARIA MARGARIDA SANTOS-SILVA, RITA SOUSA, ANA SOFIA SANTOS, PEDRO MELO, VICTOR ENCARNAÇÃO, FÁTIMA BACELLAR

RESEARCH ARTICLE

EXPERIMENTAL AND APPLIED ACAROLOGY 2006; 39(3-4):331-8

Exp Appl Acarol (2006) 39:331–338
DOI 10.1007/s10493-006-9008-3

# Ticks parasitizing wild birds in Portugal: detection of *Rickettsia aeschlimannii, R. helvetica* and *R. massiliae*

**Maria Margarida Santos-Silva · Rita Sousa · Ana Sofia Santos · Pedro Melo · Victor Encarnação · Fátima Bacellar**

Received: 17 November 2005 / Accepted: 14 May 2006 /
Published online: 3 August 2006


**Abstract** From January 2002 to December 2004, 152 ticks were collected from 40 wild birds recovered in Santo André Natural Reserve and Monsanto Forestal Park, Portugal mainland. Five ticks species were identified from 22 species of birds, and new host record were provided for some species. In addition, 32 (21%) ticks were screened by PCR to detect infections with agents belonging to order Rickettsiales: *Anaplasma phagocytophilum, Ehrlichia chaffeensis*, and *Rickettsia* spp. PCR amplicons were obtained in 5 (15.6%) tick samples. *Rickettsia* DNA exhibiting *gltA* sequences similar to those of *Rickettsia aeschilimannii, R. helvetica* and *R. massiliae* were identified in *Hyalomma marginatum, Ixodes ventalloi* and in *Rhipicephalus turanicus*, respectively. This is the first report of rickettsiae infections in ticks collected from wild birds in Portugal. Giving the results presented above wild birds play an important role in the maintenance and dissemination of several tick species and associated rickettsiae.

**Keywords** Birds · Ticks · *Rickettsia* · Portugal

M. M. Santos-Silva (✉) · R. Sousa · A. S. Santos · F. Bacellar
Centro de Estudos de Vectores e Doenças Infecciosas, Instituto Nacional de Saúde Dr. Ricardo Jorge, Avenida da Liberdade 5, 2965-575 Águas de Moura, Portugal
e-mail: m.santos.silva@insa.min-saude.pt

P. Melo
Centro de Recuperação de Animais Silvestres/Parque Florestal Monsanto, Câmara Municipal de Lisboa, Lisboa, Portugal

V. Encarnação
Instituto da Conservação da Natureza, Lisboa, Portugal

## Introduction

Wild birds are known as important reservoir of some pathogens and disseminators hosts of several arthropods, including ticks. Concerning rickettsial pathogens, birds have been described as reservoirs for *Coxiella burnetii* (Syrucek and Raska 1956) and as hosts for ticks infected with *Rickettsia sibirica* (Somov and Soldatov 1964 in Hubalek 2004) and *Anaplasma phagocytophilum* (Alekseev et al. 2001; Bjoersdorf et al. 2001; Daniels et al. 2002). In Portugal, wild birds were found parasitized by several tick species, namely *Hyalomma lusitanicum, H. marginatum, Ixodes canisuga, I. frontalis, Rhipicephalus pusillus, R. sanguineus, R. turanicus* and *Ornithodoros maritimus* (Dias 1994; Silva et al. 2001). However, there are no references about the occurrence of rickettsial infections neither on birds nor in the ticks they harbour. The aim of this work was to collect more data about tick species that parasitize wild birds in Portugal and to study the occurrence of rickettsial infections in those arthropods.

## Material and methods

Between January 2002 and December 2004, wild birds were captured in mist nets at Santo André Natural Reserve, SANR (38 °1′N, 8 °49′W) and in the Bird Rehabilitation Centers of Monsanto Forest Park, MFP (38°44′N, 9°8′W) and Quercus Santo André, QSA (38°1′N, 8°49′W). Bird identification and tick collection were done according to Nature Preservation Institute (ICN) methodologies (CEMPA/ICN 1995; Silva et al. 2001). Ticks were identified by morphological characters using standard taxonomic keys (Cordas et al. 1993; Dias 1994) and separated by species, instars and sex. Ticks that were collected alive and/or in good conditions were preserved at –80°C and processed individually for DNA extraction, as previously described (Schouls et al. 1999). Briefly, each tick was washed in 70% ethanol solution, dried and boiled for 20 min in 100µl of 0.7 M ammonium hydroxide to free the DNA. After cooling, ammonia was evaporated for 20 min at 90°C. To monitor the occurrence of false-positive samples, laboratory ticks not infected were included during DNA extraction. As positive controls we have used *A. phagocytophilum* Webster strain, *E. chaffeensis* Arkansas strain, *R. conorii*. Tick lysate was used directly for PCR. DNA amplifications were performed in a Biometra T-3 thermoblock thermal cycler employing TaqPCR master mix kit (Qiagen) according to manufacturer recommendations. Five sets of primers were used for rickettsial DNA detection according to previous descriptions: (1) Msp465f/Msp980r derived from the highly conserved regions of major surface protein-2 (*msp2*) paralogous genes of *Anaplasma phagocytophilum* (Caspersen et al. 2002); (2) ECC/ECB associated with HE1/HE3 for the amplification of *Ehrlichia chaffeensis* 16S rRNA fragment (Dawson et al. 1996); (3) RpCs.877p/RpCs.1258n and Rr190.70p/Rr190.602n, targeting the rickettsial genes for citrate synthase (*gltA*) and outer membrane protein A (*ompA*), respectively (Regnery et al. 1991). Positive PCR products were sequenced, after DNA purification by a MiniElute PCR Purification Kit (Qiagen), using an ABI automated sequencer (Applied Biosystems) according to manufacturer's instructions. Sequenciation was performed with the forward and reverse primers used for PCR identification. Sequences were identified using the BLAST software (Altschul et al. 1990, 1997).

Nucleotide sequence accession numbers

The GenBank nucleotide sequence accession numbers for partial sequences of *gltA* a gene generated in this study are for PoTiRAv3:DQ459392. For partial sequences of *gltA* and *ompA* gene generated for PoTiRAv26 are DQ459393 and DQ4459388, respectively. For PoTiRAv20 are: DQ459394 and DQ459389, respectively. For PoTiRAv25 are: DQ459395 and DQ459390, respectively. For PoTiRAv27 are: DQ459396 and DQ459391, respectively.

## Results

One hundred fifty two ticks were collected from 40 birds, belonging to 22 species. Five species of ticks were found: *Hyalomma marginatum* (111 nymphs, 7 larvae), *Ixodes ventalloi* (11 adults, 3 nymphs), *Ixodes frontalis* (3 adults, 1 nymph), *Rhipicephalus turanicus* (4 adults) and *Haemaphysalis punctata* (3 nymphs). Six ticks (3 adults, 2 nymphs, 1 larva) were grouped as *Ixodes* spp. due to the lack of body parts essential for specific identification. The instars ratio of collected ticks was 22 (14.5%) adults, 122 (80.2%) nymphs, 8 (5.3%) larvae. *H. marginatum* was the most abundant tick, infesting 77.5% (31/40) wild birds, mainly as nymphal stage. Regarding host preferences, this species also showed the widest host range, parasitizing 15 birds species. All these data are summarized in Table 1.

Of thirty-two ticks (21%) screened by PCR we were able to detect rickettsial DNA in 5 (15.6%) specimens (Table 2). Three ticks, identified as *Hyalomma marginatum* contained a rickettsia exhibiting nucleotide sequence of *gltA* 100% (347/347 bp) similar to *R. aeschlimannii* (U59722) and also the same similarity for *ompA* 100% (378/378 bp) (DQ379982). One *Rhipicephalus turanicus* was detected with rickettsial DNA exhibiting nucleotide sequence 100% similar to *R. massiliae* to both *gltA* (347/347 bp) (U59722) and *ompA* (334/334) (U43799). In one *Ixodes ventalloi* was identified a nucleotide sequence of *gltA* 100% (347/347) similar to *R. helvetica* (U59723). All ticks tested negative for the presence of DNA for *Anaplasma phagocytophilum* and *Ehrlichia chaffeensis*.

## Discussion

This study is the first report of the presence of *Haemaphysalis punctata* and *Ixodes ventalloi* parasitizing wild birds in Portugal. Additionally, is documented for the first time the occurrence of *Haemaphysalis punctata* in *Acrocephalus scirpaceus, Emberiza cirlus* and *Turdus merula; Hyalomma marginatum* in *Athene noctua, Carduelis chloris, Hirundo rustica, Lanius meridionalis, Milvus migrans, Milvus milvus, Parus caeruleus, Parus major, Passer montanus, Tyto alba* and *Turdus merula; I. frontalis* in *Asio otus; Ixodes ventalloi* in *Asio flammeus; Rhipicephalus turanicus* in *Aquila nipalensis* and *Buteo buteo*.

Most of the tick species and instars described here are commonly associated to birds. Immatures of *Hyalomma marginatum*, well known as vectors of pathogens to man and animals, are frequently transported both northward and southward by birds being one of the most recorded ticks on these host (Hoogstraal 1956, 1961; Hoogstraal and Kaiser 1961; Hoogstraal et al. 1961, 1963; Hoogstraal and

**Table 1** List of wild birds parasitized by ticks

| Bird Species | No of infested birds | Site | Tick species (number and stage) | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | *Hyalomma marginatum* | *Haemaphysalis punctata* | *Ixodes frontalis* | *Ixodes* spp.[a] | *Ixodes ventalloi* | *Rhipicephalus turanicus* |
| *Asio otus* (Long-eared owl) | 1 | MFP/QSA | | | 3F; 1N | | | |
| *Asio flammeus* (Short-eared owl) | 2 | MFP/QSA | | | | 1F; 2N; 1L | 5M; 7F; 5N | |
| *Alcedo atthis* (Kingfisher) | 2 | MFP/QSA | 15N | | | | | |
| *Athene noctua* (Little owl) | 2 | MFP/QSA | 3N | | | | | |
| *Acrocephalus scirpaceus* (Reed warbler) | 3 | SANR | 2N | 1N | | | | |
| *Aquila nipalensis* (Steppe eagle) | 1 | MFP/QSA | | | | | | 1F |
| *Bubo bubo* (Eagle owl) | 1 | MFP/QSA | 4N | | | | | |
| *Buteo buteo* (Common buzzard) | 1 | MFP/QSA | | | | | | 3F |
| *Carduelis carduelis* (Goldfinch) | 1 | MFP/QSA | | | | 1F | | |
| *Carduelis chloris* (Greenfinch) | 1 | SANR | 1N | | | | | |
| *Emberiza cirlus* (Cirl bunting) | 1 | SANR | | 1N | | | | |
| *Hirundo rustica* (Barn swallow) | 1 | SANR | 1N | | | | | |
| *Lanius meridionalis* (Iberian great grey shrike) | 2 | SANR | 19N | | | | | |
| *Milvus migrans* (Black kite) | 1 | SANR | 7N | | | | | |
| *Milvus milvus* (Red kite) | 1 | MFP/QSA | 4L | | | | | |
| *Parus caeruleus* (Blue tit) | 2 | SANR | 3N | | | | | |
| *Parus major* (Great tit) | 1 | SANR | 3N | | | | | |
| *Passer montanus* (Tree sparrow) | 2 | SANR | 2N | | | | | |
| *Saxicola communis* (Common whitethroat) | 1 | SANR | | | | 1F | | |
| *Saxicola torquata* (Common stonechat) | 6 | SANR | 28N | | | | | |
| *Turdus merula* (Black bird) | 5 | SANR | 20N | 1N | | | | |
| *Tyto alba* (Barn owl) | 2 | SANR | 3N; 3L | | | | | |
| Total (no. ticks/no. birds) | 40 | | 118/31 | 3/9 | 4/1 | 6/4 | 17/2 | 4/2 |

M—male; F—female; N—nymph; L—larvae

[a] All the *Ixodes* spp could not be attributed to any species as they were lacking parts necessary for identification

**Table 2** List of wild birds parasitized with ticks infected with rickettsial agents

| Bird species[a] | Origin | Species of tick | No. positive ticks/ no. tested | Rickettsial detection/tick instars | | |
|---|---|---|---|---|---|---|
| | | | | *Ehrlichia chaffeensis* | *Anaplasma phagocytophilum* | *Rickettsia* spp. |
| *Alcedo atthis* (OM/S) | MFP/QSA | *Hyalomma marginatum* | 1/7 | – | – | *R. aeschlimannii*/1N |
| *Athene noctua* (R/F) | MFP/QSA | *Hyalomma marginatum* | 1/1 | – | – | *R. aeschlimannii*/1N |
| *Asio flammeus* (M/F) | MFP/QSA | *Ixodes ventalloi* | 1/17 | – | – | *R. helvetica*/1M |
| *Buteo buteo* (OM/S) | MFP/QSA | *Rhipicephalus turanicus* | 1/3 | – | – | *R. massiliae*/1F |
| *Bubo bubo* (R/S) | MFP/QSA | *Hyalomma marginatum* | 1/4 | – | – | *R. aeschlimannii*/1N |
| Total | | | 5/32 | | | |

[a] (Bird status: M—migrant, OM—occasional migrant, R—resident; M—male; F—female; N—nymph/season of capture: S—Spring; F—Fall)

Aeschlimann 1982). This study corroborates that finding since *H. marginatum* was the most abundant tick and the species that showed the widest host range. In addition, these tick species might play a role in public health in Portugal, regarding rickettsial diseases. PCR testing has shown that *H. marginatum* was infected with *Rickettsia aeschlimannii*, an agent that has been recently implicated in human disease (Raoult et al. 2002). The genus *Ixodes* includes several species that are commonly or even exclusively associated with birds in Europe (Papadopoulos et al. 2001). *I. frontalis* is known to be exclusively associated with birds, which is in accordance to what we have been observing in our country. Regarding *I. ventalloi*, the bird association is not strict, although we found it on two birds. In fact, the original description of *I. ventalloi* was based on the observation of specimens collected on birds (Gil Collado 1936). The present work also describes *I. ventalloi* infection by *R. helvetica*, associated with human cases of chronic perimyocarditis (Nielsson et al. 1999). *R. helvetica* is commonly associated with *I. ricinus* ticks, which is considered the main vector implicated in human cases. The presence of this rickettsiae in alternative ticks could be attributable to the existence of secondary maintenance cycles, where agents circulate between relatively host-specific, usually non-human biting ticks and their hosts, as described for other tick-borne Rickettsiales in places where *I. ricinus* and other *Ixodes* species co-exist (Bown et al. 2003). Those additional cycles would buffer the agent from local extinction and help to re-establish the primary cycles. This hypothesis might explain our results since it has been observed that the distribution of *I. ricinus* is followed by *I. ventalloi*, in part of the country (Santos et al. 2004). In addition, the potential role of this species in public health might be considered since there are reports of human parasitism by *I. ventalloi* (Gilot and Marjolet 1982). In the literature, immatures of *Haemaphysalis punctata* are also frequently detected on Passeriformes birds, preferentially *Emberiza cirlus* (Osacar-Jimenez et al. 1998). Although rarely collected in Portugal, the presence of *H. punctata* nymphs in wild birds seems to corroborate that finding, but more birds need to be studied (especially *Emberiza cirlus*). It is worth mentioning that we have also found *Rhipicephalus turanicus* that is not commonly associated with birds. This finding could be attributable to bird's health conditions since both birds parasitized by this tick were wounded. This fact could have allowed an unusual contact with the ground which might has favoured their parasitism by *R. turanicus*. PCR assays have shown the infection of this tick species by *Rickettsia massiliae* recently shown to be pathogenic (Giustina et al. 2006).

This is the first report of rickettsiae detection in ticks collected from wild birds in Portugal, although the tick species reported here have been already described in other hosts in different regions of Portugal (Caeiro 1999; Estrada-Peña and Santos-Silva 2005). In a study conduct by Bacellar (1999), the prevalence of infection of Rickettsia like organisms (positive by hemolymph test) in different tick species was: 11.2% (33/294) in *H. marginatum*, 3.6% (13/361) in *R. turanicus*, and 100% (4/4) in *I. ventalloi*. Moreover the successful isolation of rickettsia by shell-vial was achieved in 2 (3.6%) *H. marginatum*, 1 (1.8%) *I. ventalloi* and in 6 (11%) *R. turanicus*. The strain isolates were characterized as *Rickettsia aeschlimannii, R. helvetica* and *R. massiliae*, respectively (Bacellar 1999). Here were found similar rates of rickettsial infection in the same tick species (Table 2). Nevertheless, previous studies were based only on haemocyte test and isolation, comparing with our study that only reports the detection of rickettsial pathogens by PCR. Regarding the migratory

status of birds, the occurrence of *R. helvetica* in *I. ventalloi* collected on migrating *Asio flammeus* potentially reflects the long-distance dispersion of tick-borne pathogens and the settlement of new focus. *Asio flammeus* was captured during fall migration to the south, which means that ticks and their pathogens could have originated in areas of Eastern Europe, where *R. helvetica* is commonly found. Moreover, occasional migrating *Alcedo atthis*, and *Buteo buteo* and resident *Athene noctua* and *Bubo bubo* are more likely to contribute to the maintenance and amplification of local endemic rickettsiae focus. In conclusion, this study corroborates the important role of birds in tick's dissemination and their influence on epidemiology of tick-borne rickettsiae agents in Portugal. Improvement of our knowledge about avian migration patterns and the agents that bird ticks harbour might be useful in helping to predict future outbreaks of infection due to emerging zoonotic tick-borne pathogens.

**Acknowledgements** We thank ICN technicians for their valuable contribution in the fieldwork. We also thank Liliana Vitorino for contribution in sequencing alignment and National Institute of Health for supporting this research.

## References

Alekseev AN, Dubinina HV, Semenov AV, Bolshakov CV (2001) Evidence of ehrlichiosis agents found in ticks (Acari: Ixodidae) collected from migratory birds. J Med Entomol 38:471–474

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990). Basic local alignment search tool. J Mol Biol 215:403–410

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller M, Lipman FJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25:3389–3402

Bacellar F (1999) Ticks and Rickettsiae in Portugal. In: Raoult D, Brouqui P (eds) Rickettsiae and Rickettsial Diseases. Elsevier, Paris, pp. 103–109

Bjoersdorf A, Bergstrom S, Massung RF, Haemig PD, Olsen B (2001) Ehrlichia-infected ticks on migrating birds. Emerging Infect Dis 7:877–879

Bown KJ, Begon M, Bennett M, Woldehiwet Z, Ogden NH (2003) Seasonal dynamics of *Anaplasma phagocytophila* in a rodent-tick (*Ixodes trianguliceps*) system, United Kingdom. Emerg Infect Dis 9(1):63–70

Caeiro V (1999) General review of tick species present in Portugal. Parasitol 41:11–15

Caspersen K, Park J-H, Patil S, Dumler JS (2002) Genetic variability and stability of *Anaplasma phagocytophila* msp2 (p44). Infect Immun 70(3):1230–1234

CEMPA/ ICN (1995) A anilhagem de aves na investigação científica e na gestão ambiental. Centro de Estudos de Migração e Protecção das Aves (CEMPA), Edição Portuguesa do Instituto da Conservação da Natureza (ICN), Lisboa, pp 1–24

Cordas T, Aeschlimann A, Morel PC (1993) Étude morphologique des ixodidae s. str. (Schulze, 1937) de Suisse au microscope électronique à balayage. Acarologia 34(1):21–46

Daniels TJ, Battaly GR, Liveris D, Falco RC, Schwartz I (2002) Avian reservois of the agent of human granulocytic ehrlichiosis? Emerging Infect Dis 8:1524–1525

Dawson JE, Biggie KL, Warner CK, Cookson K, Jenkins S, Levine JF, Olson JG (1996) Polymerase chain reaction evidence of *Ehrlichia chaffeensis*, an etiologic agent of human ehrlichiosis, in dogs from southeast Virginia. AJVR 57(8):1175–1179

Dias JATS (1994) As carraças (Acarina-Ixodoidea) da Península Ibérica. Algumas considerações sobre a sua Biogeografia e relacionamento com a ixodofauna Afropaleártica e Afrotropical. Instituto de Investigação Científica Tropical, Estudos Ensaios e Documentos, Lisboa, pp 1–163

Estrada-Peña A, Santos-Silva M (2005) The distribution of ticks (Acari:Ixodidae) of domestic livestock in Portugal. Exp App Acarol 36:233–246

Gil Collado J (1936) Acaros Ixodoideos de Cataluña y Baleares. Trab Mus Cienc Nat, Ser Ent 11:1–8

Gilot B, Marjolet M (1982) Contribution à l'étude du parasitisme humain par les tiques (Ixodidae et Argasidae), plus particulièrement dans le sud-est de la France. Méd Maladies Infect 12(6):340–351

Giustina V, Serafino M, Rolain J-M, Raoult D (2006) *Rickettsia massiliae* human isolation. Emerg Infect Dis 12(1):174–175

Hoogstraal H (1956) African Ixodoidea. 1. Ticks of Sudan. Bureau of Medicine and Surgery, US Navy, pp 1–1101

Hoogstraal H (1961) Migrating birds and their ectoparasites in relation to disease. East Afric Med J 38(5):221–226

Hoogstraal H, Kaiser M (1961) Ticks from European-Asiatic birds migrating through Egypt into Africa. Science 3448(133):227–278

Hoogstraal H, Kaiser M, Traylor M, Gaber S, Guindy E (1961) Ticks (Ixodoidea) on birds migrating from Africa to Europe and Asia. Bull Wld Hlth Org 24:197–212

Hoogstraal H, Kaiser M, Traylor M, Guindy E, Gaber S (1963) Ticks (Ixodidae) on birds migrating from Europe and Asia to Africa, 1959–61. Bull Wld Hlth Org 28:235–262

Hoogstraal H, Aeschlimann A (1982) Tick-host specificity. Bull Soc Entomol Suisse 55:5–32

Hubalek Z (2004) An annotated checklist of pathogenic microorganisms associated with migratory birds. J Wildlife Dis 40(4):639–659

Nilsson K, Lindquist O, Pahlson C (1999) Association of *Rickettsia helvetica* with chronic perimyocarditis in sudden death. Lancet 354:1169–1172

Osacar-Jimenez JJ, Estrada-Peña A, Lucientes-Curdi J (1998) Ticks (Acarina: Ixodidae) of wild birds in the Ebro middle basin (North-east Spain). Acarologia 39(1): 23–31

Papadopoulos B, Humair PF, Aeschlimann A, Vaucher C, Buttiker W (2001) Ticks on birds in Switzerland. Acarology 42(1):3–19

Raoult D, Fournier PE, Abboud P, Caron F (2002) First documented human *Rickettsia aeschlimannii* infection. Emerg Infect Dis 8(7):748–749

Regnery RL, Spruill CL, Plikaytis BD (1991) Genotypic identification of rickettsiae and estimation of intraspecies sequences divergence for portions of two rickettsial genes. J Bacteriol 173(5):1576–1589

Santos AS, Santos-Silva MM, Almeida VC, Bacellar F, Dumler JS (2004) Detection of *Anaplasma phagocytophilum* DNA in *Ixodes* ticks (Acari: Ixodidae) from Madeira Island and Setúbal District, Mainland Portugal. Emerg Infect Dis 10:1643–1648

Schouls LM, Van de Pol I, Rijpkema SGT, Schot CS (1999) Detection and identification of *Ehrlichia, Borrelia burgdorferi* sensu lato and *Bartonella* species in Dutch *Ixodes ricinus* ticks. J Clin Microbiol 37(7):2215–2222

Silva MM, Formosinho P, Melo P, Santos A, Filipe AR (2001) Ixodídeos (Acari: Ixodidae) parasitas de aves silváticas em Portugal. Rev Port Cienc Vet 96(540):197–199

Somov GP, Soldatov GM (1964) On the role of birds in circulation of tick-borne spotted typhus in nature. Zh Mikrobiol Epidemiol Immunobiol 1:126–129 (In Russian)

Syrucek L, Raska K (1956) Q fever in domestic and wild birds. Bull WHO 15:329–337

## 3.3. TICKS AND TICK-BORNE RICKETTSIAE SURVEILLANCE IN MONTESINHO NATURAL PARK, PORTUGAL

M. SANTOS-SILVA, R. SOUSA, A.S. SANTOS, D. LOPES, E. QUEIJO, A. DORETA, L. VITORINO, AND F. BACELLAR

SHORT REPORT

ANNALS OF NEW YORK ACADEMY OF SCIENCE 2006; 1078:137-42

# Ticks and Tick-Borne Rickettsiae Surveillance in Montesinho Natural Park, Portugal

M. SANTOS-SILVA,[a] R. SOUSA,[a] A.S. SANTOS,[a] D. LOPES,[b] E. QUEIJO,[c] A. DORETA,[b] L. VITORINO,[d] AND F. BACELLAR[a]

[a]*Centro de Estudos de Vectores e Doenças Infecciosas, Instituto Nacional de Saúde Dr. Ricardo Jorge, 2965 Águas de Moura, Portugal*

[b]*Camâra Municipal de Vinhais, Portugal*

[c]*Organização de Produtores Pecuários de Vinhais, Vinhais, Portugal*

[d]*Departamento de Biologia Vegetal/Centro de Genética e Biologia Molecular, Faculdade de Ciências, Universidade de Lisboa, Lisboa, Portugal*

ABSTRACT: **This study constitutes the first contribution to the knowledge of tick dynamics and its implication in the epidemiology of rickettsial diseases in Montesinho Natural Park (MNP), Bragança district of Portugal. Of 76 ticks collected, 12 (15.8%) were *Dermacentor (D.) marginatus*, 36 (47.4%) *D. reticulatus,* and 28 (36.8%) *Rhipicephalus (R.) sanguineus.* Isolation assays were performed by shell-vial technique on 41 ticks. Israeli spotted fever strain was an isolate from *R. sanguineus*, and three isolates of *Rickettsia slovaca* were obtained from *D. reticulatus*. All 76 ticks were screened by PCR for *Rickettsia* sp., *Ehrlichia (E.) chaffeensis,* and *Anaplasma (A.) phagocytophilum*. *Rickettsia* RpA4 strain DNA was detected in 10 *D. marginatus* and 2 *D. reticulatus*, and Israeli spotted fever strain in 1 *R. sanguineus*. No *E. chaffeensis* or *A. phagocytophilum* infection was detected. New host records are provided for *D. reticulatus*. Also described for the first time in Portugal is the isolation of *R. slovaca* from *D. reticulatus* and the isolation of Israeli spotted fever strain from *R. sanguineus*. This confirms the association of the last rickettsiae strain with the same vector tick as previously described in Israel and Sicily.**

KEYWORDS: **ticks; *Dermacentor marginatus*; *Dermacentor reticulatus*; *Rhipicephalus sanguineus*; Rickettsiae; *Rickettsia slovaca*; Israeli spotted fever; Rpa4; Portugal**

Address for correspondence: Margarida Santos-Silva, Center for Vectors and Infectious Diseases Research, National Institute of Health, Av. Liberdade 5, 2965-575 Aguas de Moura, Portugal. Voice: +351-265-912-222; fax: +351-265-912-568.
e-mail: m.santos.silva@insa.min-saude.pt

**Ann. N.Y. Acad. Sci. 1078: 137–142 (2006).** 
**doi: 10.1196/annals.1374.023**

## INTRODUCTION

Ticks are important parasites present in almost every geographic zone in the world. Several tick species are involved in the transmission of a wide range of pathogens with medical significance. In Portugal different rickettsial agents have been isolated and detected from different tick species in boutonneuse fever (BF), caused by *R. conorii*, the tick-borne disease with major implication on public health.[1] Bragança, a northeastern district of Portugal, presents the highest incidence rate of BF,[2] but no studies have been done on the prevalence of rickettsial agents in ticks from this region. As the importance of this geographical area in BF cases contrasts with the lack of information about ticks and tick-borne rickettsiae, the aim of the study was to assess tick species and pathogen interactions present in this area.

## MATERIALS AND METHODS

Ticks were collected in Montesinho Natural Park (41°47N–42°N, 6°30′W-7°12′W), Bragança district, from September to November 2004 by dragging vegetation or directly removed from hosts. All specimens were identified by morphological characters using standard taxonomic keys[3,4] and kept alive until being processed for hemolymph test as previously described.[5] Suspected ticks were directly used to rickettsiae isolation by shell-vial technique.[6] Additionally, all ticks were processed individually for DNA extraction as described.[7] PCR approaches to detect DNA from *Rickettsia (R.)* spp., *Ehrlichia (E.) chaffeensis,* and *Anaplasma (A.) phagocytophilum* were performed using five primer sets. Rickettsiae DNA detection was achieved by amplification of *gltA* fragment gene (RpCs.877p–RpCs.1258n) and *ompA* fragment gene (Rr190.70p–Rr190.602n).[8] The amplification of *E. chaffeensis* DNA was assayed by a nested PCR using ECC-ECB and HE1-HE3 primers, which amplify a fragment of the 16S rRNA gene.[9] *A. phagocytophilum* DNA was amplified by msp465f and msp980r primers, derived from the highly conserved regions of major surface protein-2 (*msp2*) paralogous genes.[10] Positive PCR products were sequenced directly using an ABI automated sequencer (Applied Biosystems USA) according to the manufacturer's instructions. Sample amplifications were performed with the forward and reverse primers used for PCR identification. Sequences were identified using the BLAST software.[11]

## RESULTS

From a total of 76 ticks collected in Montesinho Natural Park (MNP), 12 (15.8%) were *Dermacentor marginatus*, 36 (47.4%) *D. reticulatus,* and

TABLE 1. Number of ticks by species and stage collected in MNP and the detection assay results

| Month | Origin | Site | *Dermacentor marginatus* (*n*) | *Dermacentor reticulatus* (*n*) | *Rhipicephalus sanguineus* (*n*) | *Rickettsial* detection/Tick instar | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | *Anaplasma phagocytophilum* | *Ehrlichia chaffeensis* | *Rickettsia* |
| September | | | | | | | | |
| | Vegetation | Cerdeira | 5M; 2F | | | – | – | RpA4/ 5M; 2F |
| | | Trincheira | 3F | | | – | – | RpA4/ 1F |
| | | Gondosende | | 1M; 1F | | – | – | – |
| | *Canis familiaris* | Paradinha | | | 1F; 3 N | – | – | – |
| | | Paramio | | 1M; 1F | 3 N | – | – | – |
| | | Fontes de Transbaceiro | | | 1N | – | – | – |
| | | Lagarelhos | | | | – | – | – |
| | | Tuizelo | | | 18N | – | – | ISF/1N |
| | | | | | 2 N | – | – | – |
| October | | | | | | | | |
| | *Canis familiaris* | Moimenta | | 1F | | – | – | – |
| | | Quintanilha | 1F | | | – | – | RpA4/ 1F |
| | *Canis lupus* | Quintanilha | | 1F | | – | – | – |
| November | | | | | | | | |
| | *Canis familiaris* | Lagarelhos | | 1M; 3F | | | | – |
| | | Moimenta | 1F | 3 M | | | | RpA4/ 1F; 2 M |
| | | Paramio | | 1M | | | | – |
| | | Travanca | | 14 M; 7 F | | | | – |
| | | Sobreiró de Baixo | | 1 F | | | | – |
| TOTAL | | | 12 | 36 | 28 | | | |

M = male; F = female; N = nymph.

**TABLE 2. *Rickettsia* isolation results**

| Origin | Site | Tick species | No positive ticks/ no tested | *Rickettsia* isolates |
|---|---|---|---|---|
| *Canis familiaris* | Moimenta | *D. marginatus* | 0/1 | |
| | Quintanilha | | 0/1 | |
| *Canis familiaris* | Lagarelhos | *D. reticulatus* | 1/2 | *R. slovaca* |
| | Moimenta | | 0/3 | |
| | Paramio | | 0/3 | |
| | Sobreiró de Baixo | | 0/1 | |
| | Travanca | | 2/15 | *R. slovaca* |
| *Canis lupus* | Quintanilha | *D. reticulatus* | 0/1 | |
| *Canis familiaris* | Fontes de Transbaceiro | *R. sanguineus* | 0/1 | |
| | Lagarelhos | | 1/7 | ISF |
| | Paramio | | 0/1 | |
| | Paradinha | | 0/3 | |
| | Tuízelo | | 0/2 | |
| Total | | | 4/41 | |

28 (36.8%) *Rhipicephalus sanguineus*. Seasonal distribution of ticks regarding origin, site of collection, and arthropod instars are provide in TABLE 1 and FIGURE 1. Four *Rickettsia* strains were isolated. Israeli spotted fever strain (ISF) was obtained from one *R. sanguineus* (1/14), after nymph molting, and three isolates of *Rickettsia slovaca*, from adult *D. reticulatus* (3/24) as shown in TABLE 2. Of the 76 ticks tested by PCR, 83.3% *D. marginatus* (10/12), 5.6% *D. reticulatus* (2/36), and 3.6% *R. sanguineus* (1/28) were positive for the presence of rickettsial DNA using *gltA* and *OmpA* primers. Rickettsiae detected in the *Dermacentor* ticks were identified as *Rickettsia* RpA4 (similarity values between 98% and 100% for OmpA gene sequence; GenBank, AF120022). ISF was detected in one *R. sanguineus* tick (similarity values 99% for *gltA* gene sequence; GenBank, U59727), the same specimen with positive isolate, referred to above. All *D. reticulatus* positive by shell-vial for *R. slovaca* were PCR-negative, which may be on account of PCR inhibition. No *E. chaffeensis* or *A. phagocytophilum* infection was detected on ticks (TABLE 1).

## DISCUSSION

This is the first report of *D. marginatus* and *D. reticulatus* in MNP, although other studies have already documented the same species in other regions.[12] In Portugal *D. reticulatus* has a patchy distribution associated with continental climate inlands in the northeast region, with only one host known—*Canis lupus*. Here is also report the first association of *D. reticulatus* to *Canis familiaris*. In MNP the domestic dog appears to contribute not only to the maintenance of

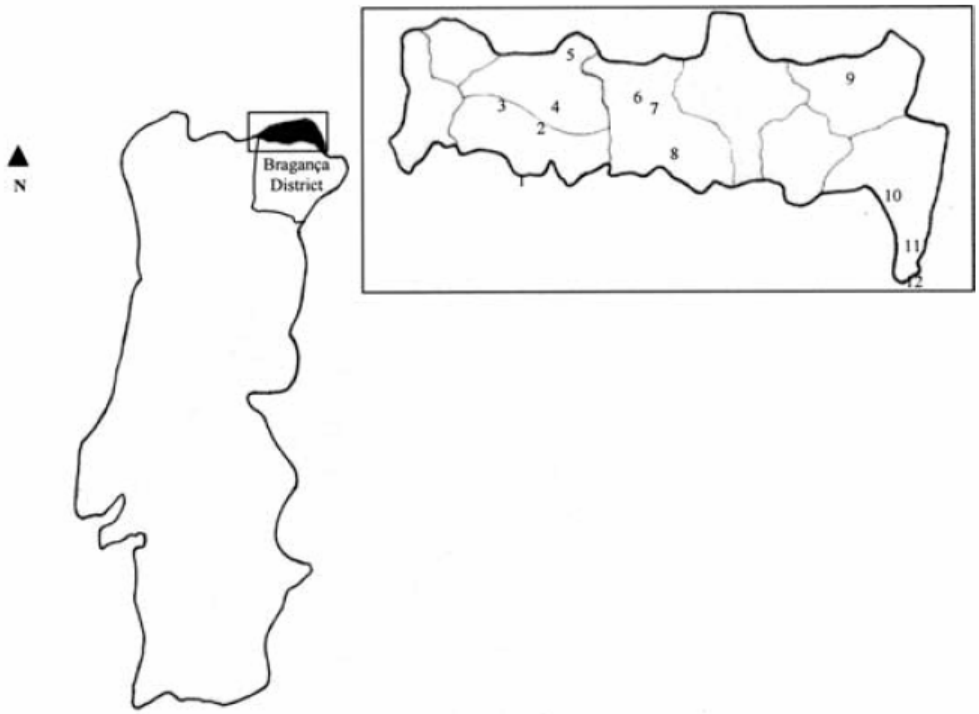



**FIGURE 1.** Montesinho Natural Park. Site of tick collection: Sobreiró de Baixo (1); Lagarelhos (2); Tuízelo (3); Travanca (4); Moimenta (5); Fontes de Transbaceiro (6); Paramio (7); Gondosende (8); Trincheira (9); Cerdeira (10); Quintanilha (11); Paradinha (12). Site of rickettsiae isolation/detection: Lagarelhos (2); Travanca (4); Moimenta (5); Trincheira (9); Cerdeira (10); Quintanilha (11).

*R. sanguineus* but represents an important host for *D. reticulatus*. This fact largely contrasts with all data recovered from other parts of the country, particularly in the south, where dogs are not involved in *D. reticulatus* cycle (Santos-Silva, unpublished data). This is the first isolation of *R. slovaca* from *D. reticulatus* and ISF strain from *R. sanguineus* in Portugal, confirming the association of this rickettsiae strain with the same vector tick as previously described in Israel and Sicily.[13] ISF isolate done after nymph molting reinforces the importance of transstadial transmission in rickettsial infectious maintenance. In Portugal *Rickettsia* RpA4 genotype has been already described in *D. marginatus* collected in the south (Vitorino *et al*. unpublished data). This study shows that *Rickettsia* RpA4 is also present in *D. reticulatus*, the same species in which it has been described for the first time in Russia.[14] The pathogenic role of this rickettsia has not yet been proven. In conclusion, the results obtained will be an important tool to develop strategies of control and prevention against ticks and tick-borne rickettsiae pathogens in Montesinho Natural Park.

## ACKNOWLEDGMENTS

This research was supported by Fundação Calouste Gulbenkian (references 65286).We thank the Montesinho Natural Park team for their valuable assistance during this study.

## REFERENCES

1. Bacellar, F. 1999. Ticks and rickettsiae in Portugal. *In* Rickettsiae and Rickettsial Diseases. D. Raoult & P. Brouqui. Eds.: 103–109 Elsevier. Paris.
2. Sousa, R. *et al*. 2003. Sobre a realidade epidemiologica da febre escaro-nodular em Portugal. Acta Medica **16:** 430–438.
3. Dias, JATS As carraças (Acarina-Ixodoidea) da Península Ibérica. Algumas Considerações Sobre A Sua Biogeografia E Relacionamento Com A Ixodofauna Afropaleártica E Afrotropical. 163 Instituto de Investigação Científica Tropical, Estudos Ensaios e Documentos, Lisboa.
4. Walker, J. *et al*. 2000.The Genus *Rhipicephalus* (Acari: Ixodidae). A Guide to the Brown Ticks of the World. Cambridge University Press. Cambridge, UK.
5. Burgdorfer, W. 1970. Hemolymph test—a technique for detection of *Rickettsiae* in ticks. Am. J. Trop. Med. Hygiene **XIX:** 1010–1014.
6. Péter, O. *et al*. 1990. Isolation by a sensitive centrifugation cell culture system of 52 strains of spotted fever group rickettsiae from ticks collected in France. J. Clin. Microbiol. **XXVIII:** 1597–1599.
7. Santos, A.S. *et al*. 2004. Detection of *Anaplasma phagocytophilum* DNA in *Ixodes* ticks (Acari: Ixodidae) from Madeira Island and Setúbal District, Mainland Portugal. Emerg. Infect. Dis. **10:** 1643–1648.
8. Regnery, R.L. *et al*. 1991. Genotypic identification of rickettsiae and estimation of intraspecies sequences divergence for portions of two rickettsial genes. J. Bacteriol. **173:** 1576–1589.
9. Dawson, J.E. *et al*. 1996. Polymerase chain reaction evidence of *Ehrlichia chaffeensis*, an etiologic agent of human ehrlichiosis, in dogs from southeast Virginia. Am. J. Vet. Res. **57:** 1175–1179.
10. Caspersen, K. *et al*. 2002. Genetic variability and stability of *Anaplasma phagocytophila* msp2 (p44). Infect. Immun.**70:** 1230–1234.
11. Altschul, S.F. *et al*. 1990. Basic local alignment search tool. J. Mol. Biol. **215:** 403–410.
12. Caeiro, V. 1999. General review of tick species present in Portugal. Parassitology **41:** 11–15.
13. Giammanco, G.M. *et al*. 2003. Israeli spotted fever Rickettsia in Sicilian *Rhipicephalus sanguineus* ticks. Emerg. Infect. Dis. **9:** 892–893.
14. Shpynov, S. *et al*. 2004. Detection of a *Rickettsia* closely related to *Rickettsia aeschlimannii*, "*Rickettsia heilongjiangensis*", *Rickettsia* sp. strain RpA4, and *Ehrlichia muris* in ticks collected in Russia and Kazakhstan. J. Clin. Microbiol. **42:** 2221–2223.

## 3.4. DETECTION OF *RICKETTSIA HELVETICA* AND OTHER SPOTTED FEVER GROUP RICKETTSIAE IN *IXODES RICINUS* FROM TAPADA NACIONAL DE MAFRA, PORTUGAL

SANTOS AS, SANTOS-SILVA MM, DE SOUSA R, FORMOSINHO P, NÚNCIO MS, BACELLAR F

SHORT REPORT

IN SUBMISSION

# Detection of *Rickettsia helvetica* and other spotted fever group rickettsiae in *Ixodes ricinus* from Tapada Nacional de Mafra, Portugal

Santos AS[1], Santos-Silva MM[1], De Sousa R[1], Formosinho P[2], Núncio MS[1], Bacellar F[1]

[1]Centro de Estudos de Vectores e Doenças Infecciosas, Instituto Nacional de Saúde Dr. Ricardo Jorge, Lisboa, Portugal;
[2]Laboratório Privado de Análises Clínicas, Lisboa, Portugal.

Corresponding author: Ana Sofia Pereira dos Santos. Centro de Estudos de Vectores e Doenças Infecciosas, Instituto Nacional de Saúde Dr. Ricardo Jorge, Edifício LEMES, Avenida Padre Cruz, 1649-016 Lisboa, Portugal;
Tel: +351 217 508 125; Fax: +351 217 508 521
e-mail: ana.santos@insa.min-saude.pt

ABSTRACT:

The presence of tick-borne rickettsial agents in *Ixodes ricinus* ticks was investigated in a densely infested Portuguese recreational park, Tapada Nacional de Mafra. A total of 998 *Ixodes ricinus* were tested by PCR, individually or in pools, for detection of *Rickettsia* spp., *Ehrlichia chaffeensis* and *Anaplasma phagocytophilum*. *Rickettsia* DNA exhibiting sequences similar to those of *Rickettsia helvetica,* and *R. monacensis* strains IRS3 and IRS4 were identified. This is the first report of *I. ricinus* infection by other spotted fever rickettsiae beside *R. helvetica* in Portugal. Further consideration should be given to *I. ricinus* as vector of several rickettsiae whose pathogenicity to humans is still unknown.

RUNNING TITLE

*Rickettsia* spp. in *Ixodes ricinus*, Portugal

KEY WORDS: *Ixodes ricinus*; Spotted fever group rickettsiae; *Rickettsia helvetica*; IRS3; IRS4; *gltA*; *OmpA; OmpB*; Portugal.

INTRODUCTION:

*Ixodes ricinus* are among the most important European ticks regarding Public Health concerns. It is a well-known vector of several agents causing diseases in humans, such as tick-borne encephalitis virus (TBE), *Borrelia burgdorferi s.l.* (Lyme borreliosis), *Anaplasma phagocytophilum* (human granulocytic anaplasmosis), *Babesia divergens* and *B. microti* (human babesiosis) (Estrada-Pena & Jongejan, 1999). It is also responsible for the transmission of *Rickettsia helvetica* recently associated to cases of febrile illness (Fournier *et al.*, 2000). *I. ricinus* is regarded as a hygrophilic species presenting in Portugal a patchy distribution influenced mainly by environmental determinants, such as climate, landscape fragmentation and composition (Baptista, 2006). Regardless, in some areas of the country with adequate conditions this tick species can be

abundant as in the case of *Tapada Nacional de Mafra* (*TNM*). This national park with more than 800 ha is a densely wooded territory located in the Central Western Coast of Portugal (38º 56'N 9º 17'W). It was originally created as a hunting area, but it is also used as a recreational and educational park. *TNM* supports a diverse wildlife population composed of birds, reptiles, and small to large mammals, including rodents, insectivores, carnivores, lagomorphs, and artiodactyls (wild boars, fallow deer and elks). The availability of such diverse vertebrate hosts helps to maintain an abundant tick's population, dominated by *I. ricinus* but also including other *Ixodes*, *Dermacentor*, *Haemaphysalis*, and *Rhipicephalus* species (MM Santos-Silva, unpublished data). Given the repeated contact between humans and ticks in *TNM*, the occurrence of tick-borne agents with potential Public Health importance has been a matter of concern for the park's administration which has promoted the realization of several studies to increase knowledge regarding this subject. The occurrence of a great variety of *B. burgdorferi s.l.* in ticks, including *B. afzelii*, *B. garinii*, *B. lusitaniae*, and *B. valaisiana* has already been documented (Baptista *et al.*, 2004, 2006). However, no studies have yet addressed the occurrence of other tick-borne agents, such as those belonging to the Order Rickettsiales. In this study, the presence of *Anaplasma phagocytophilum, Ehrlichia chaffeensis,* and *Rickettsia* spp. was investigated in *I. ricinus* ticks from *TNM*.

## MATERIALS AND METHODS:

***Tick sampling***. During 4 day-trials in February 2002, a total of 1064 ticks were collected from *TMN*, including: 998 *I. ricinus*, 29 *Haemaphysalis inermis*, 28 *H. puntacta* and 9 *Dermacentor marginatus*. This work details only the study regarding *I. ricinus* ticks. The majority of *I. ricinus* (132 males, 130 females and 710 nymphs) was collected when questing for hosts, by means of flagging vegetation in 4 sampling areas. These areas are designated feeding places for the large animal population, and were selected for hosting a large number of animals daily. Additional specimens were collected from *Dama dama*, captured during the hunting season (9 males and 17 females). Most arthropods were processed in groups pooled by sex, stage, and sampling area comprising 36 pools of adult ticks (ranging from 2 to 20 arthropods) and 6 pools of immatures (ranging from 55 to 200 arthropods). Approximately 9% of the questing adults collected in each sampling area were processed individually, representing a total of 24 ticks (12 males and 12 females). The ticks were

washed in alcohol 70%, passed through distillate water and dried in filter paper. Using a sterile pestle per sample, ticks were homogenized in 400µl PBS supplemented with 10% fetal calf serum (Gibco, Invitrogen, UK). Half sample homogenate was used for DNA extraction and the other 200µl was stored at –80ºC.

*Polymerase chain reaction (PCR).* Genomic DNA was extracted using DNeasy Tissue Kit, according to the manufacture's instructions (Qiagen GmbH, Germany). To monitor the occurrence of false-positive samples, homogenates of laboratory-reared ticks were included during extraction as negative controls (one control per each extraction run containing 5 tick samples). Several sets of primers were used for Rickettsiales detection, including: i) GE9f/GE10r for the amplification of *A. phagocytophilum* 16S rRNA (*rrs*) gene (Chen *et al*., 2004) and MSP465f/MSP980r, for the amplification of a hypervariable region of *A. phagocytophilum* surface protein genes (Caspersen *et al*., 2002); ii) ECC/ECB followed by HE1/HE3, outer and inner primer pair, respectively, targeting *E. chaffeensis rrs* (Dawson *et al*., 1996); iii) RpCs415/RpCs1220 and Rr190.70p/Rr190.602n, which target a fragment of *Rickettsia* spp. genes encoding for citrate synthase (*gltA*) and outer membrane protein A (*ompA*), respectively (Regnery *et al*., 1991). Due to the lack of *ompA* gene in some *Rickettsia* spp., to complement these later screening all *gltA* positive samples that were not amplified with *ompA* primers were re-tested with OF/OR pair, for the amplification of the alternative outer membrane protein B (*ompB*) gene (Choi *et al*., 2005). Primer sequences and amplification conditions have been previously described (Regnery *et al*., 1991; Chen *et al*., 1994; Dawson *et al*., 1996; Choi *et al*., 2005; Caspersen *et al*., 2002). PCR were performed in Biometra T3 thermoblock thermal cycler (Biometra GmbH, Germany) using a total volume of 50µl master mix containing 1 µM of each primer, 2.5 U of Taq DNA polymerase, 20mM of each dNTP, 10 mM Tris-HCL, 1.5 mM $MgCl_2$, 50 mM KCl and 10µl DNA extract (1µl in nested reactions). As PCR negative controls we have used DNA extracted from arthropods reared in a laboratory tick colony and as positive controls *R. conorii* Malish strain, *A. phagocytophilum* Webster strain and *E. chaffeensis* Arkansas strain.

*Sequencing.* DNA amplicons from positive samples were purified (Jetquick PCR Purification Kit; Genomed GmbH, Germany) and sequenced using an ABI automated sequencer (Applied Biosystems). After manual review and editing, sequence homology searches were performed using BLASTn and the NCBI nr nucleotide database.

Nucleotide sequence accession numbers: The GenBank nucleotide sequence accession numbers for partial sequences generated in this study are: DQ910783 (PoTiR1dt), DQ910784

(PoTiR2dt), DQ910785 (PoTiR3dt), and EF051167 (PoTiR4dt) for *gltA* gene; DQ910781 (PoTiR1dt), DQ910782 (PoTiR2dt) and EF053275 (PoTiR4dt) for *ompA* gene; EU126928 (PoTiR3dt) for *ompB*.

RESULTS:

Seven out of 42 pools and 1 out of 24 individually processed *I. ricinus*, tested positive for rickettsiae. Positive amplicons were obtained only from questing ticks and the sequencing results are presented in table 1. DNA from female I-7 (named PoTiR1dt) and pool 26 (named PoTiR2dt) exhibited nucleotide sequence 99% similar to spotted fever *Rickettsia* strain IRS3 to both *gltA* (372/373 bp) (AF140706) and *ompA* (497/499) (AF141909) genes. Pool 1 (named PoTir3dt) contained rickettsial DNA exhibiting nucleotide sequences 99% similar in both *gltA* and *ompB* (positions 361/363 bp and 495/499 bp, respectively) when compared to *R. helvetica* sequences available in GenBank (U59723 and AF123725, respectively). The tick pool 4 (named, PoTiR4dt) presented DNA with a 100% homology to *R. monacensis* (IRS4) in both *gltA* (373/373) (DQ100163) and *ompA* (499/499) (DQ100169) sequences. Additionally, 4 other pools (P31-33) revealed appropriate amplicons with *gltA* and *ompA* fragments of 376-bp and 534-bp, respectively. However, DNA sequencing of these PCR products showed ambiguous bases in the sequence, probably resulting from polling samples and they were excluded from further analysis. Neither *A. phagocytophilum* nor *E. chaffeensis* DNA was detected in this study.

DISCUSSION:

In Europe several agents belonging to the order Rickettsiales have been associated to *I. ricinus*. *A. phagocytophilum* is now reported in several countries and cases of human disease associated to this agent are especially documented in Northern and Central Europe (Strle, 2004). *Rickettsia helvetica*, initially described in Switzerland as the Swiss agent, was the first rickettsia to be isolated from this tick species (Beati *et al*., 1994). More recently, *R. monacensis* (previous known as IRS4) was isolated from ticks collected in a public park in Munich, Germany (Simser *et al*., 2002). Other yet-to-be-cultured rickettsiae have also been detected by PCR testing, such as the Cadiz agent in southwestern Spain and IRS3 in Slovakia (Márquez *et al*., 1998; Sekeyova *et*

*al.*, 2000), probably representing variant genotypes of the *R. monacensis*. So far *R. helvetica* is the only rickettsiae species transmitted by *I. ricinus* that has been associated to human disease after its detection in French patients with febrile illness (Fournier *et al*., 2000).

In Portugal, both *A. phagocytophilum* and *R. helvetica* has been already identified in *I. ricinus* (Bacellar, 1999; Santos *et al.*, 2004), but no cases of human infection by these Rickettsiales have been definitely proven. Additionally, the description of a case fulfilling the serological criteria for *E. chaffeensis* infection (David de Morais *et al.*, 1991), suggested the occurrence of this agent in our country. Since the vector of *E. chaffeensis* is largely unknown in Europe, it was considered of interest to include this agent's screening in the present study. The results presented herein evidenced the occurrence of *R. helvetica*, IRS3 and IRS4 in *TNM*. The detection of *R. helvetica* in an area such as *TNM*, where the exposure to tick infested habitats is highly promoted, could have direct implication in Public Health and thus deserves further investigation. Moreover, the association of IRS3 and IRS4 to *I. ricinus*, a tick species that frequently bites humans, should also merit attention. Regarding the other Rickettsiales in study, no active infections could be identified in the analysed ticks. Given the presence of *A. phagocytophilum* in other Portuguese areas where *Ixodes* spp. are known to occur, the present results may be justified by the patchy seasonal and spatial distribution that usually characterizes this agent.

Further investigation should be planed in *TNM* to investigate aspects such as the absence of *A. phagocytophilum*, rickettsiae prevalence, occurrence of co-infection in ticks and the rickettsiae species involved in human diseases in the area.

ACKNOWLEDGEMENTS

This research was partially supported by *Instituto Nacional de Saúde Dr. Ricardo Jorge* grant BIC DT/13/2000 and through the *Fundação para a Ciência e a Tecnologia* grant BD/8610/2002. We thank Ricardo Alves, Director of *TNM* and all staff for their valuable contribution in the fieldwork. We also thank Sónia Pedro and Ana Cardoso for their assistance in sequencing.

REFERENCES:

1. BACELLAR FC. Ticks and Spotted Fever Rickettsiae in Portugal. *In*: Rickettsiae and Rickettsial Diseases at the Turn of the Third Millenium. Raoult D, Brouqui P, editors. Paris: Elsevier; 1999a. p. 423-7.

2. BAPTISTA S. [Lyme borreliosis in Portugal. Study on vector(s), agent(s) and risk factors]. Tese de Doutoramento, Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa, Lisboa, 2006. [PhD Thesis in English].

3. BAPTISTA S, QUARESMA A, AIRES T, KURTENBACH K, SANTOS-REIS M, NICHOLSON M, COLLARES-PEREIRA M. Lyme borreliosis spirochetes in questing ticks from mainland Portugal. Int J Med Microbiol. 2004;293(37):109-16.

4. BEATI L, HUMAIR PF, AESCHLIMANN A, RAOULT D. Identification of spotted fever group rickettsiae isolated from *Dermacentor marginatus* and *Ixodes ricinus* ticks collected in Switzerland. Am J Trop Med Hyg. 1994;51:138-48.

5. CASPERSEN K, PARK J-H, PATIL S, DUMLER JS. Genetic variability and stability of *Anaplasma phagocytophila msp2* (*p44*). Infect Immun. 2002;70(3):1230-4.

6. CHEN SM, DUMLER JS, BAKKEN JS, WALKER DH. Identification of granulocytotropic *Ehrlichia* species as the etiologic agent of human disease. J Clin Microbiol. 1994;32:589-95.

7. CHOI YJ, JANG WJ, RYU JS, LEE SH, PARK KH, PAIK HS, KOH YS, CHOI MS, KIM IS. Spotted fever group and typhus group rickettsioses in humans, South Korea. Emerg Infect Dis. 2005;11(2):237-44.

8. DAVID DE MORAIS JD, DAWSON JE, GREENE C, FILIPE AR, GALHARDAS LC, BACELLAR F. First European cases of ehrlichiosis. Lancet. 1991;338:633–4.

9. DAWSON JE, BIGGIE KL, WARNER CK, COOKSON K, JENKINS S, LEVINE JF, OLSON JG. Polymerase chain reaction evidence of *Ehrlichia chaffeensis*, an etiologic agent of human ehrlichiosis, in dogs from southeast Virginia. AJVR. 1996;57(8):1175-9.

10. ESTRADA-PENA A, JONGEJAN F. Ticks feeding on humans: a review of records on human-biting Ixodoidea with special reference to pathogen transmission. Exp Appl Acarol. 1999;23(9):685-715.

11. FOURNIER PE, GRUNNENBERGER F, JAULHAC B, GASTINGER G, RAOULT D. Evidence of *Rickettsia helvetica* infection in humans, eastern France. Emerg Infect Dis. 2000;6:389-92.

12. MARQUEZ FJ, MUNIAIN MA, SORIGUER RC, IZQUIERDO G, RODRÍGUEZ-BAÑO J, BOROBIO MV. Genotypic identification of an undescribed spotted fever group rickettsia in *Ixodes ricinus* from Southwestern Spain. Am J Trop Med Hyg. 1998;58(5):570-7.

13. REGNERY RL, SPRUILL CL, PLIKAYTIS BD. Genotypic identification of rickettsiae and estimation of intraspecies sequences divergence for portions of two rickettsial genes. J Bacteriol. 1991;173(5):1576-89.

14. SANTOS AS, SANTOS-SILVA MM, ALMEIDA VC, BACELLAR F, DUMLER JS. Detection of *Anaplasma phagocytophilum* DNA in *Ixodes* ticks (Acari:Ixodidae) from Madeira Island and Setúbal District, Mainland Portugal. Emerg Infect Dis. 2004;10(9):1643-8.

15. SEKEYOVÁ Z, FOURNIER PE, REHACEK J, RAOULT D. Characterization of a new Spotted Fever Group rickettsia detected in *Ixodes ricinus* (Acari: Ixodidae) collected in Slovakia. J Med Entomol. 2000;37(5):707-13.

16. SIMSER, JA, PALMER, AT, FINGERLE, V, WILSKE, B, KURTTI TJ, MUNDERLOH UG. *Rickettsia monacensis* sp. nov., a spotted fever group *Rickettsia* from ticks (*Ixodes ricinus*) collected in a European city park. Appl Environ Microbiol. 2002;68(9):4559-66.

17. STRLE F. Human granulocytic ehrlichiosis in Europe. Int J Med Microbiol. 2004;293(Suppl 37):27-35.

TABLE 1 – Tick samples detected with *Rickettsia* DNA

| Sample[a] | Tested ticks stage[b](origin)[c] | PCR results | | | | | *Rickettsia* homology (genotype designation) |
|---|---|---|---|---|---|---|---|
| | | *A. phagocytophilum* | | *E. chaffeensis* | *Rickettsia* spp. | | |
| | | *rrs* | *msp2* | *rrs* | *gltA* | *OmpA* (*OmpB)* | |
| I-7 | 1F(V) | - | - | - | + | + | IRS3 (PoTiR1dt) |
| P1 | 10M(V) | - | - | - | + | (+) | *R. helvetica* (PoTiR3dt) |
| P4 | 10M(V) | - | - | - | + | + | *R. monacensis*/IRS4 (PoTiR4dt) |
| P26 | 20F(V) | - | - | - | + | + | IRS3 (PoTiR2dt) |
| P30 | 100N(V) | - | - | - | + | + | |
| P31 | 100N(V) | - | - | - | + | + | |
| P32 | 200N(V) | - | - | - | + | + | |
| P33 | 200N(V) | - | - | - | + | + | |

[a]I individually processed arthropods, P pool of ticks; [b]M male, F female, N nymph; [c]V ticks collected in vegetation (questing ticks).

## 3.5. PCR-BASED SURVEY OF *ANAPLASMA PHAGOCYTOPHILUM* IN PORTUGUESE TICKS (ACARI: IXODIDAE)

SANTOS AS, SANTOS-SILVA MM, SOUSA R, BACELLAR F, DUMLER JS

RESEARCH ARTICLE

IN SUBMISSION

# PCR-based survey of *Anaplasma phagocytophilum* in Portuguese ticks (Acari: Ixodidae)

Santos AS[1], Santos-Silva MM[1], Sousa R[1], Bacellar F[1], Dumler JS[2]

[1]Centro de Estudos de Vectores e Doenças Infecciosas, Instituto Nacional de Saúde Dr. Ricardo Jorge, Lisboa, Portugal;
[2]Division of Medical Microbiology, Department of Pathology, The Johns Hopkins University School of Medicine, Baltimore, Maryland, USA.

Corresponding author: Ana Sofia Pereira dos Santos. Centro de Estudos de Vectores e Doenças Infecciosas, Instituto Nacional de Saúde Dr. Ricardo Jorge, Edifício LEMES, Avenida Padre Cruz, 1649-016 Lisboa, Portugal;
Tel: +351 217 508 125; Fax: +351 217 508 521
e-mail: ana.santos@insa.min-saude.pt

ABSTRACT

A total of 2006 ticks, collected in Madeira Island and seven districts of Mainland Portugal, were examined by polymerase chain reaction (PCR) for the presence of *Anaplasma phagocytophilum*. Active infections were detected exclusively in *Ixodes* species, including six questing *I. ricinus* nymphs from Madeira Island, one questing *I. ventalloi* nymph from Setúbal District, and two *I. ventalloi* adults found parasitizing domestic cats in both Setúbal and Santarém District. These findings confirm prior observations and suggest the persistence of *A. phagocytophilum* on Madeira Island, where its presence was first documented in 2000. Moreover, it adds *I. ventalloi* and domestic cats to the list of potential elements of the agent's enzootic cycles in Portugal. Molecular analysis of PCR amplicons suggests the existence of two *A. phagocytophilum* genotypes in Portugal, one of which is identical or very similar to North American strains implicated in human disease.

RUNNING TITLE

*Anaplasma phagocytophilum* in Portuguese ticks

KEYWORDS

Ticks; Tick-borne agent; *Anaplasma phagocytophilum*; Polymerase chain reaction (PCR); Portugal;

INTRODUCTION

*Anaplasma phagocytophilum* (formerly *Ehrlichia phagocytophila*, *E. equi* and the human granulocytic ehrlichiosis agent [HGE agent]) (Dumler *et al*., 2001) is an obligate intracellular bacterium that is well established as a worldwide tick-borne agent of veterinary importance and is also currently considered an emerging human pathogen in both US and Europe (Strle, 2004; Dumler, 2005; Lotric-Furland *et al*., 2006). The disease in humans is known as human granulocytic anaplasmosis (HGA), formerly human granulocytic ehrlichiosis, and presents clinically as a non-

specific febrile illness accompanied by hematological abnormalities and hepatic injury. Severe and fatal infections have been occasionally reported, especially in US (Bakken *et al*., 1994, 1996; Hardalo *et al*., 1995; Jahangir *et al*., 1998). The main route of *A. phagocytophilum* transmission is via infected tick salivary secretions during a tick-bite. Several ticks species in the *Ixodes persulcatus*/*ricinus* complex are considered primary vectors for this agent, including *I. scapularis* and *I. pacificus*, respectively in Eastern and Western parts of North America, *I. ricinus* in Western, Central and Northern Europe (Macleod & Gordon, 1933; Richter *et al*., 1996; Telford *et al*., 1996) and *I. persulcatus* in Western Europe and Asia (Cao *et al*., 2000, 2006; Morozova *et al*., 2002; Kim *et al*., 2003; Wen *et al*., 2003; Rar *et al*, 2005).

When this study was initiated in 2002, limited information was available regarding the presence of *A. phagocytophilum* in Portugal, except for Madeira Island that described its presence in *I. ricinus* (Núncio *et al*., 2000). Thus a broader study was undertaken to investigate the occurrence of *A. phagocytophilum* in Portuguese ticks and to further characterize the agent by molecular approaches. It was integrated with other research activities of Center for Vector and Infectious Diseases Research, National Institute of Health Dr. Ricardo Jorge (*CEVI*/*INSA*), in a joint effort to promote improved knowledge of tick-borne agents potentially involved in human disease in the country. The present report summarizes all available data regarding *A. phagocytophilum* detection in ticks collected by active surveillance in Portugal during 2002-2006.

## MATERIAL AND METHODS

Ticks sampling. During 2002 to 2006, ticks were collected in several areas of Bragança, Braga, Leiria, Lisboa, Portalegre, Santarém, and Setúbal Districts, mainland Portugal and on Madeira Island, Madeira Archipelago. The areas were selected for the field-trials, either because *Ixodes* species were known to be present, or because specific interest in a given tick-borne disease was expressed for integration with ongoing projects. Detailed information regarding 1,384 ticks was previously described (Santos *et al*., 2004; Santos-Silva *et al*., 2006a, 2006b; Santos *et al*., in submission). The remaining 622 ticks included in this study were obtained either from additional field work or collected during the above mentioned surveys (Santos *et al*., 2004; Santos *et al*., in submission). Ticks were obtained by flagging vegetation or were directly removed from wild and domestic animals. Some additional specimens found parasitizing humans and sent to our

laboratory by medical institutions or directly removed from persons who presented at *CEVDI/INSA* were also included in this study. All specimens were identified to the species level by standard taxonomic keys (Cordas *et al*., 1993; Dias, 1994), and either immediately processed or frozen at -80ºC until used.

DNA extraction and polymerase chain reaction (PCR). Ticks were processed individually, or in some samples obtained from Lisboa District, pooled according to feeding condition, stage, sex, and site of collection, as described elsewhere (Santos *et al*., in submission). Genomic DNA was extracted from individual ticks by crude digestion of tissues in 0.7 mol/L ammonium hydroxide at 100°C, as described by Schouls and coworkers (1999). DNA from pooled samples was extracted from 200 µl of tick homogenates prepared in PBS using the DNeasy Tissue Kit, according to the manufacturer's instructions (Qiagen GmbH, Germany). To monitor the occurrence of cross-contamination, laboratory-reared ticks were included during extraction as negative controls (one control per each extraction run containing 6 to 23 tick samples). The presence of *A. phagocytophilum* DNA in tick samples was screened by PCR or nested PCR using different sets of primers: i) MSP465f/MSP980r derived from the highly conserved 5' region of major surface protein-2 (*msp2*) paralogous genes (Caspersen *et al*., 2002); ii) GE9f/GE10r which amplify a 919 bp fragment of the 16S rRNA gene (*rrs*) (Chen *et al*., 1994); iii) HS1/HS6 followed by HS43/HS45 for the amplification of a 442-bp sequence of heat-shock operon, *groESL* (Sumner *et al*., 1997). Both *msp2* and *rrs* primers were used for the initial screening of part of the samples, as described elsewhere (Santos *et al*., 2004; Santos *et al.*, in submission). The rest of the ticks presented here were screened using *msp2* testing alone. All samples that tested positive with the *msp2* and *rrs* screening during 2002-2006 were further analysed using *groESL* primers. PCR was performed in a total 50 µl volume containing 1 µM of each primer, 2.5 U of Taq DNA polymerase, 200 mM of each dNTP, 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl and 5 to 10 µl of DNA template (1 µl in nested reactions). Primer sequences and PCR conditions were previously described (Chen *et al*., 1994; Sumner *et al*., 1997; Caspersen *et al*., 2002). In each PCR run, known positive and negative samples (extraction controls and water) were included as controls. DNA amplicons from positive samples were purified (Jetquick PCR Purification Kit; Genomed GmbH, Germany) and sequenced using an ABI automated sequencer (Applied Biosystems). After manual review and editing, sequence homology searches were performed using BLASTn using the NCBI nr nucleotide database.

GenBank accession numbers. The accession numbers of *A. phagocytophilum* 16S rRNA partial gene (*rrs*) sequences obtained in this study are: EU098006 for ticks nos. 118 and 122; EU098007 for ticks nos. 160 and 246. The *A. phagocytophilum groESL* partial sequences are: EU004826 for ticks nos. 93 and 118; EU004827 for ticks nos. 160 and 246.

## RESULTS

A total of 2006 ticks belonging to 14 species were collected, including 117 *Dermacentor marginatus*, 76 *D. reticulatus*; 6 *Hyalomma lusitanicum*, 12 *H. marginatum*, 29 *Haemaphysalis inermis*, 34 *H. punctata*, 2 *Ixodes acuminatus*, 1 *I. bivari*, 5 *I. hexagonus*, 1202 *I. ricinus*, 144 *I. ventalloi*, 49 *Rhipicephalus bursa*, 77 *R. pusillus* and 252 *R. sanguineus group* (Figure 1, Table 1-2). PCR testing was performed either on individually processed ticks (n=1032) or using pooled ticks (n=974, divided in 42 tick samples), representing a total of 1074 DNA samples. Of the 320 samples initially screened by both *msp2* and *rrs* primers, *A. phagocytophilum* DNA was detected in 8 samples, corresponding to 6 questing *I. ricinus* nymphs collected from Madeira Island, previously referred as nos. 11, 60, 118, 122, and 137 (Santos *et al*., 2004) and 1 questing *I. ventalloi* nymph and 1 male *I. ventalloi* collected from a domestic cat, both obtained from Setúbal District and referred as nos. 160 and 246, respectively (Santos *et al*., 2004). All 8 positive samples were identified by *msp2* primers, although *rrs* PCR failed to amplify DNA in 3 of these ticks. The standard amplification of *rrs* can be limited by low sensitivity owing to the presence of a single copy in each bacterial genome. In contrast *msp2* is a member of a multigene family composed as many as 105 paralogs per genome, which enhances detection sensitivity (Scorpio *et al*., 2004). Because of the higher sensitivity of PCR for *msp2* compared to *rrs*, the remaining study samples (712 DNA samples) were screened only using *msp2* DNA testing. Of these, one additional sample was found positive for *A. phagocytophilum*, corresponding to an *I. ventalloi* female collected from a domestic cat in Santarém District (no. 348). Additional amplifications with the *groESL* primers were performed in 4 *A. phagocytophilum msp2* and *rrs* positive samples (nos. 93, 118, 160, 246).

Partial sequences of *msp2* obtained from all 9 ticks showed between 85.7% to 98.5% similarity, between 86.9% and 94.2% similarity when compared to representative *msp2* sequences from North American *A. phagocytophilum* strains isolated from humans (Webster strain - Wisconsin and USG3 strain - eastern US), and between 35.0% and 63.7% similarity compared to outgroup *A.*

*marginale msp2* and *msp3* sequences, respectively (Figure 1A). Of interest, the sequences obtained from *I. ventalloi* from mainland Portugal clustered together and separate from other *msp2* sequences obtained from *I. ricinus* on Madeira Island (Figure 1A). When amplified using *rrs* primers, compared to other US *A. phagocytophilum* strains obtained from humans (U02521,Wisconsin strain and CAHU–HGE1 and CAHU–HGE2, California strains), sequences were 100% (783/783) identical for the *I. ventalloi* nos. 160 and 246 and *I. ricinus* no. 118 and 100% (645/645) identical for *I. ricinus* no 93 (Figure 1B). Moreover, the *groESL* partial sequences were also identical for the two *I. ricinus* (nos. 93 and 118) and two *I. ventalloi* (nos. 160 and 246) showing 100% (463/463) and 97.8% (453/463) similarity to the Californian strains, CAHU-HGE1 and CAHU-HGE2. Although the *rrs* failed to provide any evidence of differences between the mainland and Madeira Island strains, the *groESL* analysis reinforced the differences observed in *msp2* sequences of *A. phagocytophilum* strains derived from *I. ricinus* and *I. ventalloi* ticks (Figure 1C).

## DISCUSSION

These data confirm, in actively questing *I. ricinus* collected from Madeira Island, the presence of *A. phagocytophilum* that is closely related to the North American strains involved in human disease. *I. ricinus* is regarded as a hygrophilic tick that presents a patchy distribution in Portugal mainly influenced by environmental determinants, such as climate, landscape composition and fragmentation (Baptista, 2006). Regardless, in areas with favorable conditions, as is the case for Madeira Island and Mafra (Lisboa District, mainland), it can be the dominant tick species (Almeida, 1996; Baptista, 2006). Moreover, *I. ricinus* is also frequently found parasitizing humans in Portugal (MM Santos-Silva, unpublished data) and their potential for transmitting pathogenic agents is illustrated by the occurrence of at least one *Ixodes*-borne disease, Lyme borreliosis caused by the bacteria *Borrelia burgdorferi s.l.*, which has been included in the list of national notifiable diseases since 1999 (Lopes de Carvalho & Núncio, 2006). Thus, one can assume that the presence of *A. phagocytophilum* in *I. ricinus* on Madeira Island presents a potential health threat to both humans and animals that needs further investigation.

These data also illustrate that *A. phagocytophilum* might not be uniformly distributed in our country. For example in Mafra (Lisboa District), an area where *I. ricinus* are abundant no infected

ticks were found. In contrast, on Madeira Island, *A. phagocytophilum* seems to be endemic given the detection of infected *I. ricinus* in surveys conducted at two different times (Núncio *et al*., 2000; Santos *et al*., 2004). This reflects either continuous introduction of the agent by migrating birds and/or the existence of vertebrate hosts that sustain the agent's active cycles, again likely rewarding subjects for further investigation.

Another ecologically interesting finding was the identification of *A. phagocytophilum* in *I. ventalloi* ticks either collected from vegetation in Setúbal District or parasitizing cats in both Setúbal and Santarém District. Besides the major vectors of the *I. persulcatus*/*ricinus* complex species, the involvement of other *Ixodes* ticks in natural enzootic cycles of *A. phagocytophilum* is already reported, such as with *I. spinipalpis* in Colorado (Zeidner *et al*., 2000), *I. dentatus* on the New England Coast (Goethert & Telford, 2003) and possibly *I. trianguliceps* in England (Bown *et al*., 2003). Although the direct role of these ticks in human and domestic animal diseases still needs to be evaluated, it seems to be limited due to their relative host specificity and restricted questing potential. When compared with members of *I. persulcatus*/*ricinus* complex, *I. spinipalpis, I. dentatus,* and *I. trianguliceps* are regarded as important bridge-elements, maintaining parallel transmission cycles that could provide support for primary vectored infectious agents (Zeidner *et al.*, 2000; Bown *et al.*, 2003; Goethert & Telford, 2003). This is a possible explanation for the results obtained here, although the vector competency of *I. ventalloi* for *A. phagocytophilum* transmission has yet to be demonstrated.

Of interest is the fact that *msp2* and *groESL* sequences of *A. phagocytophilum* derived from *I. ventalloi* were closely related to each other and divergent from those obtained in *I. ricinus*. The *A phagocytophilum* HZ strain genome has at least 105 copies of the *msp2* (or *p44*) gene multifamily and *msp2* primers were designed to target the highly conserved 5' flanking sequences detected in a sample of about 30 of these copies (Caspersen *et al*., 2002). The *msp2* possesses 5' and 3' conserved sequences that flank a hypervariable region that recombines to generate antigenically diverse bacterial populations. Thus, *msp2* is best known for diversity and not for its potential application as a molecular phylogenetic or epidemiological tool. Yet, given the high degree of nucleotide conservation surrounding the *msp2* hypervariable region, its application for molecular phylogeny is likely valid, and the agreement with *groESL* results supports the hypothesis that distinct *A. phagocytophilum* lineages exist in Portugal.

Since 2 of the 3 infected *I. ventalloi* ticks were obtained from domestic cats, a potential role is suggested for these animals in agent's maintenance cycles. In fact, domestic cats are on the list

of vertebrate hosts affected by *A. phagocytophilum* infections in both US and Europe (Bjoerdorff *et al*., 1999; Lappin *et al*., 2004; Shaw *et al*., 2005). Additionally, a study performed by Levin and coworkers (2002) also describes the presence of active infection in feral cats captured in Connecticut, US. Although no clinical evaluation was undertaken in the current study, the cat that harboured the infected ticks in Setúbal District was apparently healthy but free-roaming; no information was available regarding the Santarém District cat. Additionally, *I. ventalloi* has also been found parasitizing wild birds and several orders of mammals, such as Carnivora, Erinaceomorpha, Lagomorpha, Rodentia, and Soricomorpha (Dias *et al*., 1994; Santos-Silva *et al*., 2006b). So far, no cases of human parasitism by this species are reported in Portugal, although *I. ventalloi* has been found on humans in France (Gilot & Marjolet, 1982). Thus, the enzootic cycles of this *A. phagocytophilum* strain may involve several wild animals and the pathogenic potential for both humans and domestic animals should be evaluated.

It is also worth mentioning the lack of *A. phagocytophilum* detection in non-*Ixodes* ticks, such as in a 500 $m^2$ site in Baixa de Palmela, Setubal District where positive *Ixodes* ticks were found, and the analysis of several other tick species revealed no *A. phagocytophilum*. In this site during the morning flagging performed on April 2003, 1 positive *I. ventalloi* was collected with 13 *R. pusillus* and 5 *R. sanguineus* group (data not detailed). These results are in accordance with several other studies that link *A. phagocytophilum* to the genus *Ixodes* and only sporadically to other arthropods, such as *Dermacentor variabilis*, *Dermacentor occidentalis* and *Haemaphysalis leporispalutris* in US (Goethert & Telford, 2003; Holden *et al*., 2003), *Haemaphysalis longicornis* in Korea (Kim *et al*., 2003), *Hyalomma detritum* in Tunisia (Sarih *et al*., 2005), *Dermacentor silvarum* in China (Cao *et al*., 2006), the Trombiculidae mite *Neotrombicula autumnalis* in Spain (Fernandez-Soto *et al*., 2001), Syringophilidae quill mites *Torotrogla merulae and Syringophilopsis sturni* in Poland (Skoracki *et al*., 2006). The majority of these arthropods were collected while feeding on vertebrate hosts. Thus, it is possible that these reports represent the passive acquisition of *A. phagocytophilum* infected blood and not the capacity for reproduction or transmission. Regarding *H. leporispalutris* and *D. variabilis*, experimental studies demonstrate that although these ticks are susceptible to infection, they are unable to maintain the agent transstadially and to successfully transmit it to a vertebrate host, respectively, and are therefore considered incompetent vectors (Des Vignes *et al*., 1999; Goethert & Telford, 2003).

In summary, the results of this 4-year study of Portuguese ticks show that *A. phagocytophilum* occurs in *Ixodes* species from both mainland and Madeira Island and as such,

the potential exists for both human and animal infections. Moreover, these molecular analyses document the existence of two *A. phagocytophilum* genotypes in Portugal, one of which is identical or very similar to North American strains implicated in human cases of disease.

ACKNOWLEDGEMENTS

This research was partially supported by the Portuguese government through the *Fundação para a Ciência e a Tecnologia* grant BD/8610/2002. We thank the staff of the *Unidade Laboratorial de Utilização Comum*, *Instituto Nacional de Saude Dr. Ricardo Jorge*, for their valuable assistance with sequencing.

REFERENCES:

1. ALMEIDA, V. [Ixodídeos da zona planaltica do Paul da Serra. Contribuição ao estudo da sua ecologia]. Méd Vet. 1996;46:5-22. [Article in Portuguese]
2. BAKKEN JS, DUMLER JS, CHEN SM, ECKMAN MR, VAN ETTA LL, WALKER DH. Human granulocytic ehrlichiosis in the upper Midwest United States: a new species emerging? JAMA. 1994;272:212–8.
3. BAKKEN JS, KRUETH J, WILSON-NORDSKOG C, TILDEN RL, ASANOVICH K, DUMLER JS. Clinical and laboratory characteristics of human granulocytic ehrlichiosis. JAMA. 1996;275:199–20.
4. BAPTISTA S. [Lyme borreliosis in Portugal. Study on vector(s), agent(s) and risk factors]. Tese de Doutoramento, Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa, Lisboa, 2006. [PhD Thesis in English].
5. BJOERSDORFF A, SVENDENIUS L, OWENS JH, MASSUNG RF. Feline granulocytic ehrlichiosis-a report of a new clinical entity and characterisation of the infectious agent. J Small Anim Pract. 1999;40(1):20-4.
6. BOWN KJ, BEGON M, BENNETT M, WOLDEHIWET Z, OGDEN NH. Seasonal dynamics of *Anaplasma phagocytophila* in a rodent-tick (*Ixodes trianguliceps*) system, United Kingdom. Emerg Infect Dis. 2003;9(1):63-70.

7. CAO WC, ZHAN L, HE J, FOLEY JE, DE VLAS SJ, WU XM, YANG H, RICHARDUS JH, HABBEMA JD. Natural *Anaplasma phagocytophilum* infection of ticks and rodents from a forest area of Jilin Province, China. Am J Trop Med Hyg. 2006;75(4):664-8.

8. CAO, WC, ZHAO QM, ZHANG PH, DUMLER JS, ZHANG XT, FANG LQ, YANG H. Granulocytic ehrlichiae in *Ixodes persulcatus* ticks from an area in China where Lyme disease is endemic. J Clin Microbiol. 2000;38:4208–10.

9. CASPERSEN K, PARK J-H, PATIL S, DUMLER JS. Genetic variability and stability of *Anaplasma phagocytophila msp2* (*p44*). Infect Immun. 2002;70(3):1230-4.

10. CHEN S-M, DUMLER JS, BAKKEN JS, WALKER DH. Identification of a granulocytotropic *Ehrlichia* species as the etiologic agent of human disease. J Clin Microbiol. 1994;32:589-95.

11. CORDAS T, AESCHLIMANN A, MOREL PC. Étude morphologique des ixodidae s. str. (Schulze, 1937) de Suisse au microscope électronique à balayage. Acarologia. 1993;34(1):21-46.

12. DES VIGNES F, LEVIN ML, FISH D. Comparative vector competence of *Dermacentor variabilis* and *Ixodes scapularis* (Acari: Ixodidae) for the agent of human granulocytic ehrlichiosis. J Med Entomol. 1999;36:182–5.

13. DIAS J. In: [As carraças (Acarina-Ixodoidea) da Península Ibérica. Algumas considerações sobre a sua Biogeografia e relacionamento com a ixodofauna Afropaleártica e Afrotropical]. Estudos Ensaios e Documentos. Lisboa: Instituto de Investigação Científica Tropical; 1994, pp 1-163. [Book in Portuguese].

14. DIAS JA, NÚNCIO MS, GONÇALVES A. [Contribuição para a elaboração de um inventário da fauna ixodoideológica (Acarina-Ixodoidea) de Portugal]. Garcia de Orta. 1994;20(1-2):49-68. [Article in Portuguese and abstract in English]

15. DUMLER JS. *Anaplasma* and *Ehrlichia* infection. Ann NY Acad Sci. 2005;1063:361-73.

16. DUMLER JS, BARBET AF, BEKKER CPJ, DASCH GA, PALMER GH, RAY SC, RIKIHISA Y, RURANGIRWA FR. Reorganization of genera in the families Rickettsiaceae and Anaplasmataceae in the order Rickettsiales: unification of some species of *Ehrlichia* with *Anaplasma*, *Cowdria* with *Ehrlichia* and *Ehrlichia* with *Neorickettsia*, description of six new species combinations and designation of *Ehrlichia equi* and 'HGE agent' as subjective synonyms of *Ehrlichia phagocytophila*. Int J Syst Evol Microbiol. 2001;51:2145-65.

17. FERNANDEZ-SOTO P, PEREZ-SANCHEZ R, ENCINAS-GRANDES A. Molecular detection of *Ehrlichia phagocytophila* genogroup organisms in larvae of *Neotrombicula autumnalis* (Acari: Trombiculidae) captured in Spain. J Parasitol. 2001;87(6):1482-3.

18. GILOT B, MARJOLET M. Contribution à l'étude du parasitisme humain par les tiques (Ixodidae et Argasidae), plus particulièrement dans le sud-est de la France. Méd Maladies Infect. 1982;12(6):340-51.

19. GOETHERT HK, TELFORD SR 3RD. Enzootic transmission of the agent of human granulocytic ehrlichiosis among cottontail rabbits. Am J Trop Med Hyg. 2003;68(6):633-7.

20. HARDALO CJ, QUAGLIARELLO V, DUMLER JS. Human granulocytic ehrlichiosis in Connecticut: report of a fatal case. Clin Infect Dis. 1995;21(4):910-4.

21. HOLDEN K, BOOTHBY JT, ANAND S, MASSUNG RF. Detection of *Borrelia burgdorferi*, *Ehrlichia chaffeensis*, and *Anaplasma phagocytophilum* in ticks (Acari: Ixodidae) from a coastal region of California. J Med Entomol. 2003;40(4):534-9.

22. JAHANGIR A, KOLBERT C, EDWARDS W, MITCHELL P, DUMLER JS, PERSING DH. Fatal pancarditis associated with human granulocytic ehrlichiosis in a 44-year-old man. Clin Infect Dis. 1998;27(6):1424-7.

23. KIM CM, KIM MS, PARK MS, PARK JH, CHAE JS. Identification of *Ehrlichia chaffeensis*, *Anaplasma phagocytophilum*, and *A. bovis* in *Haemaphysalis longicornis* and *Ixodes persulcatus* ticks from Korea. Vector Borne Zoonotic Dis. 2003;3(1):17-26.

24. LAPPIN MR, BREITSCHWERDT EB, JENSEN WA, DUNNIGAN B, RHA JY, WILLIAMS CR, BREWER M, FALL M. Molecular and serologic evidence of *Anaplasma phagocytophilum* infection in cats in North America. J Am Vet Med Assoc. 2004;225(6):893-6.

25. LEVIN ML, NICHOLSON WL, MASSUNG RF, SUMNER JW, FISH D. Comparison of the reservoir competence of medium-sized mammals and *Peromyscus leucopus* for *Anaplasma phagocytophilum* in Connecticut. Vector Borne Zoonotic Dis. 2002;2(3):125-36.

26. LOPES DE CARVALHO I, NÚNCIO MS. Laboratory diagnosis of Lyme borreliosis in Portuguese National Institute of Heath (1990-2004). Eurosurveillance. 2006;11(10):257-60.

27. LOTRIC-FURLAN S, ROJKO T, PETROVEC M, AVSIC-ZUPANC T, STRLE F. Epidemiological, clinical and laboratory characteristics of patients with human granulocytic anaplasmosis in Slovenia. Wien Klin Wochenschr. 2006;118(21-22):708-13.

28. MACLEOD J, GORDON WS. Studies on tick-borne fever in sheep I: transmission by the tick *Ixodes ricinus* and descripition of the disease produced. Parasitology. 1933;25:273-83.

29. MOROZOVA OV, DOBROTVORSKY AK, LIVANOVA NN, TKACHEV SE, BAKHVALOVA VN, BEKLEMISHEV AB, CABELLO FC. PCR detection of *Borrelia burgdorferi sensu lato*, tick-borne encephalitis virus, and the human granulocytic ehrlichiosis agent in *Ixodes*

*persulcatus* ticks from Western Siberia, Russia. J Clin Microbiol. 2002;40(10):3802-4.

30. NÚNCIO MS, SCHOULS LM, VAN DE POL I, BACELLAR F, FILIPE A. The role of *Ixodes ricinus* as a vector of zoonotic agents in Madeira Island. In: Proceedings of the Seventh Bienal Meeting of European Tick Study Group, Oxford, United Kingdom, 2000 April 28.

31. RAR VA, FOMENKO NV, DOBROTVORSKY AK, LIVANOVA NN, RUDAKOVA SA, FEDOROV EG, ASTANIN VB, MOROZOVA OV. Tickborne pathogen detection, Western Siberia, Russia. Emerg Infect Dis. 2005;11(11):1708-15.

32. RICHTER PJ, KIMSEY RB, MADIGAN JE, BARLOUGH JE, DUMLER JS, BROOKS DL. *Ixodes pacificus* (Acari: Ixodidae) as a vector of *Ehrlichia equi* (Rickettsiales: Ehrlichieae). J Med Entomol. 1996;33(1):1–5.

33. SANTOS-SILVA M, DE SOUSA R, SANTOS AS, LOPES D, QUEIJO E, DORETA A, VITORINO L, BACELLAR F. Ticks and tick-borne rickettsiae surveillance in Montesinho Natural Park, Portugal. Ann NY Acad Sci. 2006a;1078:137-42.

34. SANTOS-SILVA MM, DE SOUSA R, SANTOS AS, MELO P, ENCARNAÇÃO V, BACELLAR F. Ticks parasitizing wild birds in Portugal: detection of *Rickettsia aeschlimanii*, *R. helvetica* and *R. massiliae*. Exp Appl Acarol. 2006b;39:331-8.

35. SANTOS AS, SANTOS-SILVA MM, ALMEIDA VC, BACELLAR F, DUMLER JS. Detection of *Anaplasma phagocytophilum* DNA in *Ixodes* ticks (Acari:Ixodidae) from Madeira Island and Setúbal District, mainland Portugal. Emerg Infect Dis. 2004;10(9):1643-8.

36. SANTOS AS, SANTOS-SILVA MM, DE SOUSA R, FORMOSINHO P, NÚNCIO MS, BACELLAR F. Detection of *Rickettsia helvetica* and other spotted fever group rickettsiae in *Ixodes ricinus* from Tapada Nacional de Mafra, Portugal. In submission.

37. SARIH M, M'GHIRBI Y, BOUATTOUR A, GERN L, BARANTON G, POSTIC D. Detection and identification of *Ehrlichia* spp. in ticks collected in Tunisia and Morocco. J Clin Microbiol. 2005;43(3):1127-32.

38. SCHOULS LM, VAN DE POL I, RIJPKEMA SG, SCHOT CS. Detection and identification of *Ehrlichia*, *Borrelia burgdorferi sensu lato*, and *Bartonella* species in Dutch *Ixodes ricinus* ticks. J Clin Microbiol. 1999;37(7):2215-22.

39. SCORPIO DG, AKKOYUNLU M, FIKRIG E, DUMLER JS. CXCR2 blockade influences *Anaplasma phagocytophilum* propagation but not histopathology in the mouse model of human granulocytic anaplasmosis. Clin Diagn Lab Immunol. 2004;11(5):963-8.

40. SHAW SE, BINNS SH, BIRTLES RJ, DAY MJ, SMITHSON R, KENNY MJ. Molecular evidence of tick-transmitted infections in dogs and cats in the United Kingdom. Vet Rec. 2005;157(21):645-8.

41. SKORACKI M, MICHALIK J, SKOTARCZAK B, RYMASZEWSKA A, SIKORA B, HOFMAN T, WODECKA B, SAWCZUK M. First detection of *Anaplasma phagocytophilum* in quill mites (Acari: Syringophilidae) parasitizing passerine birds. Microbes Infect. 2006;8(2):303-7.

42. STRLE F. Human granulocytic ehrlichiosis in Europe. Int J Med Microbiol. 2004;293(Suppl 37):27-35.

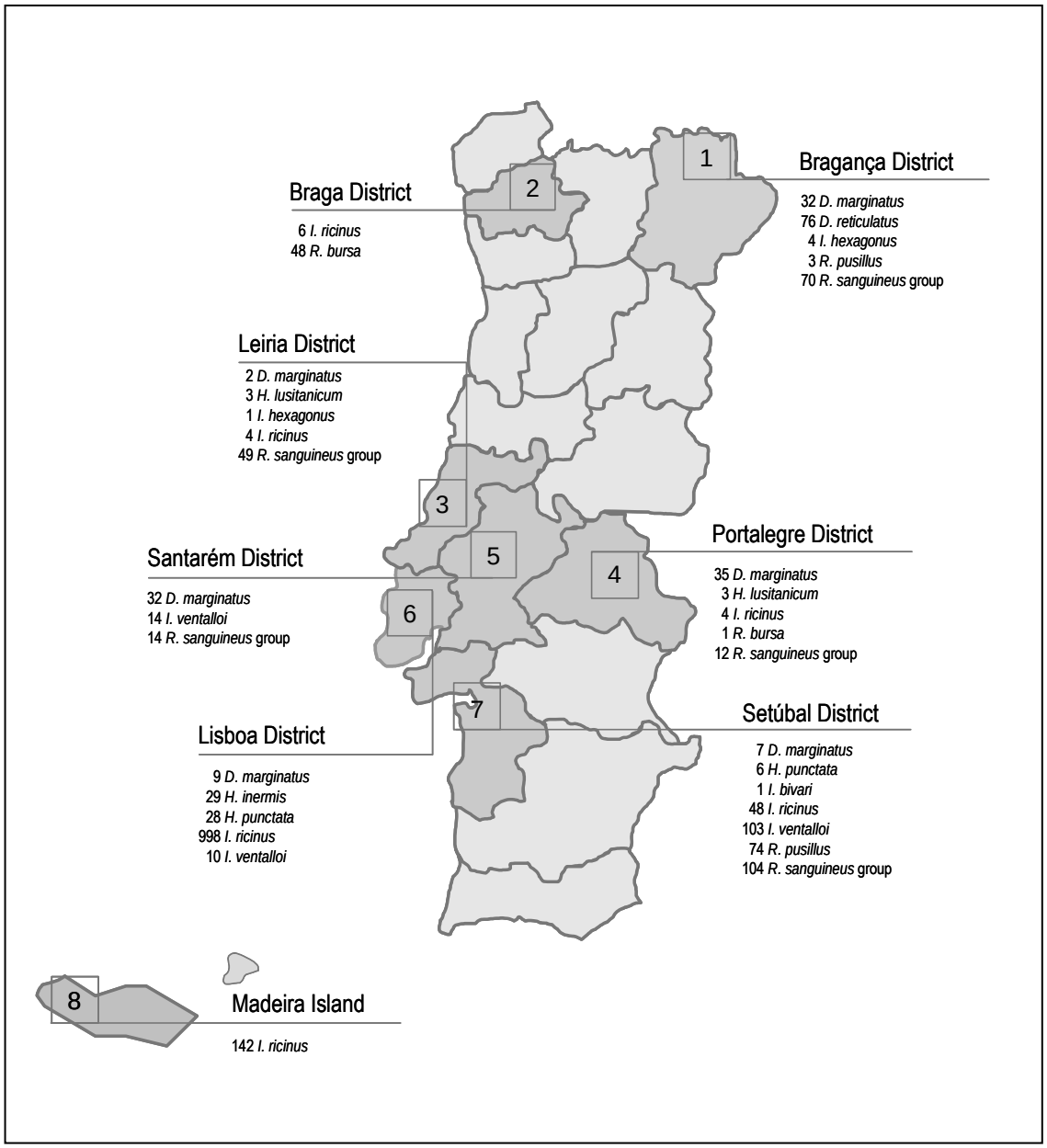
43. SUMNER JW, NICHOLSON WL, MASSUNG RF. PCR amplification and comparison of nucleotide sequences from the *groESL* heat shock operon of *Ehrlichia* species. J Clin Microbiol. 1997;35(8):2087-92.

44. TELFORD SR 3RD, DAWSON JE, KATAVOLOS P, WARNER CK, KOLBERT CP, PERSING DH. Perpetuation of the agent of human granulocytic ehrlichiosis in a deer tick-rodent cycle. Proc Natl Acad Sci USA. 1996;93(12):6209-14.

45. WEN B, CAO W, PAN H. Ehrlichiae and ehrlichial diseases in China. Ann N Y Acad Sci. 2003;990:45-53.

46. ZEIDNER NS, BURKOT TR, MASSUNG R, NICHOLSON WL, DOLAN MC, RUTHERFORD JS, BIGGERSTAFF BJ, MAUPIN GO. Transmission of the agent of human granulocytic ehrlichiosis by *Ixodes spinipalpis* ticks: evidence of an enzootic cycle of dual infection with *Borrelia burgdorferi* in Northern Colorado. J Infect Dis. 2000;182(2):616-9.

FIGURE 1. Tick species* and collection areas on the mainland Portugal and in the Madeira Archipelago (2002-2006).




*Ticks species in study: *Dermacentor marginatus, D. reticulatus, Haemaphysalis inermis, H. puntacta, Hyalomma lusitanicum, Ixodes bivari, I. hexagonus, I. ricinus, I. ventalloi, Rhipicephalus bursa, R. pusillus, R. sanguineus* group;
From a total of 2006 ticks, 34 could not be ascribed to any specific collection area.

TABLE 1. *A. phagocytophilum* PCR results from ticks collected in vegetation, according to sampling area, species and gender.

| Area | Ticks species* | No tested†‡ | No of positives†‡ | Sample code |
|---|---|---|---|---|
| 1 | *D. marginatus* | (5M, 5F)[a] 1M, 1F | - | |
| | *D. reticulatus* | (1M, 1F)[a] 2M, 1F | - | |
| | *R. sanguineus* group | 8M, 8F | - | |
| 2 | *I. ricinus* | 3N | - | |
| 3 | *D. marginatus* | 2F | - | |
| | *H. lusitanicum* | 2M, 1F | - | |
| | *I. ricinus* | 3F, 1M | - | |
| | *R. sanguineus* group | 24M, 21F | - | |
| 4 | *D. marginatus* | 14M, 21F | - | |
| | *H. lusitanicum* | 2M, 1F | - | |
| | *R. bursa* | 1F | - | |
| | *R. sanguineus* group | 5M, 7F | - | |
| | *I. ricinus* | 2M, 1F | - | |
| 5 | *D. marginatus* | 14M, 18F | - | |
| | *R. sanguineus* group | 7M, 7F | - | |
| 6 | *D. marginatus* | 3M, 6F | - | |
| | *H. inermis* | 26M, 3F | - | |
| | *H. puntacta* | 9M, 19F | - | |
| | *I. ricinus* | (12M, 12F)[b] | - | |
| | | (120M, 118F, 710N)[b, pool] | | |
| 7 | *D. marginatus* | 6M, 1F | - | |
| | *H. puntacta* | 2M, 1F, 3N | - | |
| | *I. ricinus* | (21M, 19F, 3N)[c] | - | |
| | *I. ventalloi* | (36M, 19F, 28N)[c] | (1N)[c] | 160 |
| | *R. pusillus* | 27M, 47F | - | |
| | *R. sanguineus* group | 56M, 44F | - | |
| 8 | *I. ricinus* | (1M, 2F, 139N)[c] | (6N)[c] | 11; 60; 93; 118; 122; 137 |

*Ticks species in study*: Dermacentor marginatus, D. reticulatus, Haemaphysalis inermis, H. puntacta, Hyalomma lusitanicum, Ixodes ricinus, I. ventalloi, Rhipicephalus bursa, R. pusillus, R. sanguineus group;*

†M male, F female, N nymph;

‡Ticks in parentheses were previously processed for *A. phagocytophilum* testing and described elsewhere: [a]Santos-Silva *et al*., 2006a; [b]Santos *et al*., in submission; [c]Santos *et al*., 2004;

[pool]ticks were processed in pools according to sampling area, stage and gender, comprising 30 pools of adult ticks (ranging from 2 to 20 arthropods) and 6 pools of immatures (ranging from 55 to 200 arthropods).

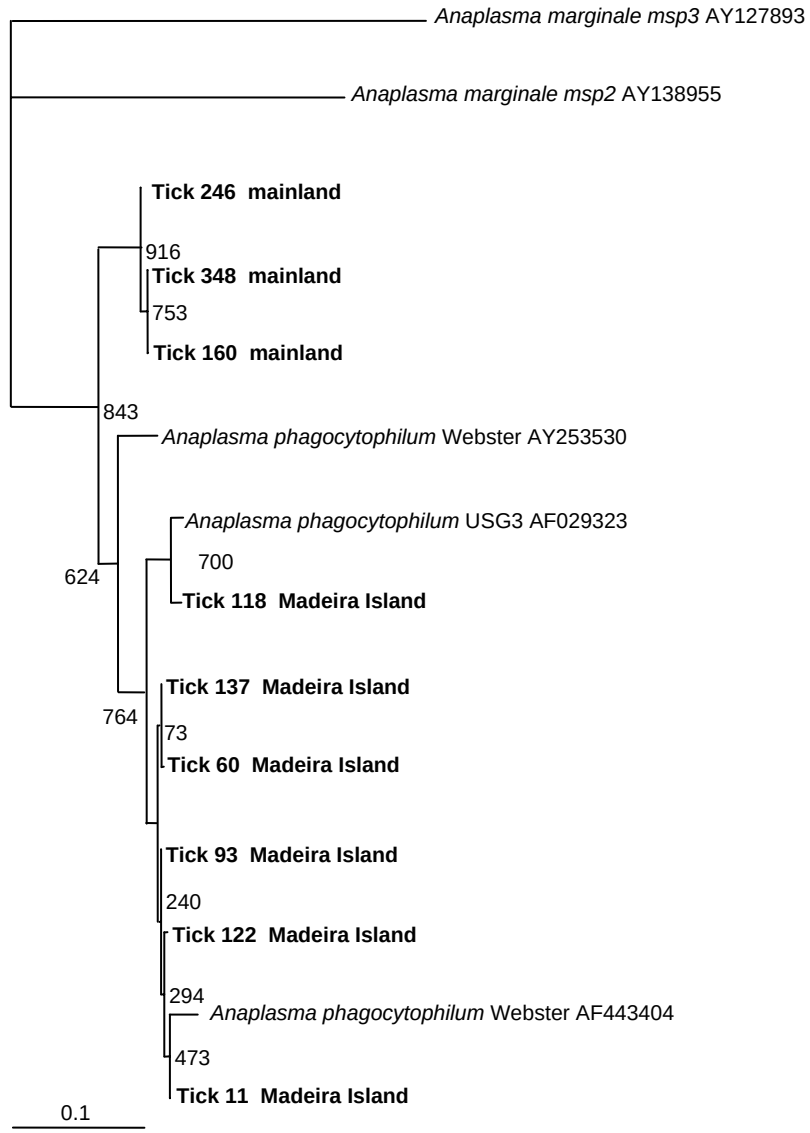
TABLE 2. *A. phagocytophilum* PCR results from ticks collected parasitizing vertebrate hosts, according to sampling area, species and gender.

| Host | Ticks Species* | No tested per area†‡ | No of positives†‡ | Sample code |
|---|---|---|---|---|
| Wild birdsΨ | | | | |
| *Alcedo athis* | *H. marginatum* | Area NA - (7N)[a] | - | |
| *Athene noctua* | *H. marginatum* | Area NA - (1N)[a] | - | |
| *Asio flammeus* | *I. ventalloi* | Area NA - (5M, 7F, 5N)[a] | - | |
| *Bubo bubo* | *H. marginatum* | Area NA - (4N)[a] | - | |
| *Buteo buteo* | *R. sanguineus* group | Area NA - (3F)[a] | - | |
| Feral mammals | | | | |
| *Canis lupus* | *D. reticulatus* | Area 1 - (1F)[b] | - | |
| | *I. ricinus* | Area 2 – 1N | - | |
| *Capreolus Capreolus* | *I. ricinus* | Area 2 – 1F | - | |
| *Dama dama* | *I. ricinus* | Area 6 - (9M, 17F)[c, pool] | - | |
| *Meles meles* | *I. hexagonus* | Area 3 - 1F | - | |
| *Mustela putoris* | *I. acuminatus* | Area NA - 2F | - | |
| Domestic mammals/ Men | | | | |
| *Canis familiaris* | *D. marginatus* | Area 1 - (2F)[b] 5M, 6F | - | |
| | *D. reticulatus* | Area 1- (20M, 13F)[b] 19M, 15F | - | |
| | *I. hexagonus* | Area 1- 1F, 3N | - | |
| | *R. sanguineus* group | Area 1 - (1F, 27N)[b] 4M, 4F | - | |
| | *R. pusillus* | Area 1 - 1M, 2F | - | |
| *Capra hircus* | *D. marginatus* | Area 1- 1M, 1F | - | |
| | *R. sanguineus* group | Area 1 1M, 1F Area 3 1M, 3F | - | |
| *Equs caballos* | *R. bursa* | Area 2 - 29M, 19F | - | |
| *Felis catus* | *I. bivari* | Area 7 - 1F | - | |
| | *I. ventalloi* | Area 5 14F; Area 6 4M, 6F; | Area 5 1F | 348 |
| | | Area 7 (4M, 6F)[d] 2M, 8F | Area 7 (1M)[d] | 246 |
| *Ovis aires* | *D. marginatus* | Area 1 - 2M, 3F | - | |
| | *D. reticulatus* | Area 1 - 1M, 2F | - | |
| | *R. sanguineus* group | Area 1 - 8M, 8F | - | |
| *Homo sapiens* | *I. ricinus* | Area 2 1N; Area 4 1F; Area 7 5F | | |
| | *R. sanguineus* group | Area 7 - 1F, 3N | | |

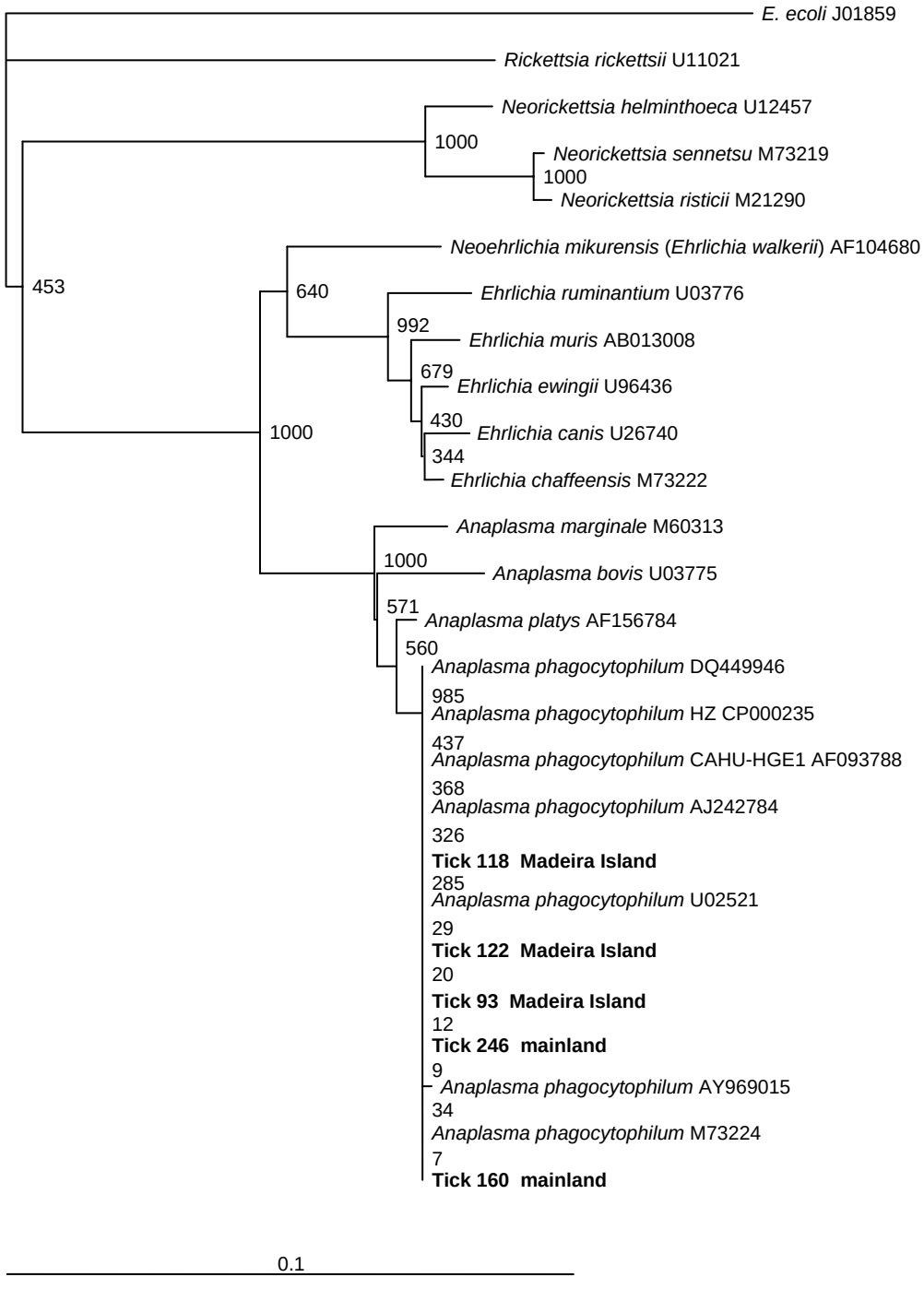
*Ticks species in study: *Dermacentor marginatus, D. reticulatus, Hyalomma marginatum, Ixodes acuminatus, I. bivari, I.hexagonus, I. ricinus, I. ventalloi, Rhipicephalus sanguineus group;*
†M male, F female, N nymph;
‡Ticks in parentheses were previously processed for *A. phagocytophilum* testing and described elsewhere: [a]Santos-Silva *et al*., 2006b; [b]Santos-Silva *et al*., 2006a; [c]Santos *et al*., in submission; [d]Santos *et al.*, 2004; NA Ticks that could not be ascribed to any specific collection area;
ΨBirds in study were found wounded and sent to 2 rehabilitation centres located in Setúbal and Lisboa Dstrict, respectively. The geographic area of capturing was missing for all animals.
[pool]Ticks were processed in pools comprising 6 pools of adult ticks (ranging from 2 to 10 arthropods), according to the animal sampled and ticks gender.

FIGURE 2- Dendrograms showing the phylogenetic relationships between *A. phagocytophilum* sequences presented in this study and other Anaplasmataceae based on partial nucleotide sequences of the *msp2* (A), *rrs* (B), and *groESL* (C). Bootstrap values (out of 1000 iterations) are shown at the nodes. Bar = substitutions/1000 bp.

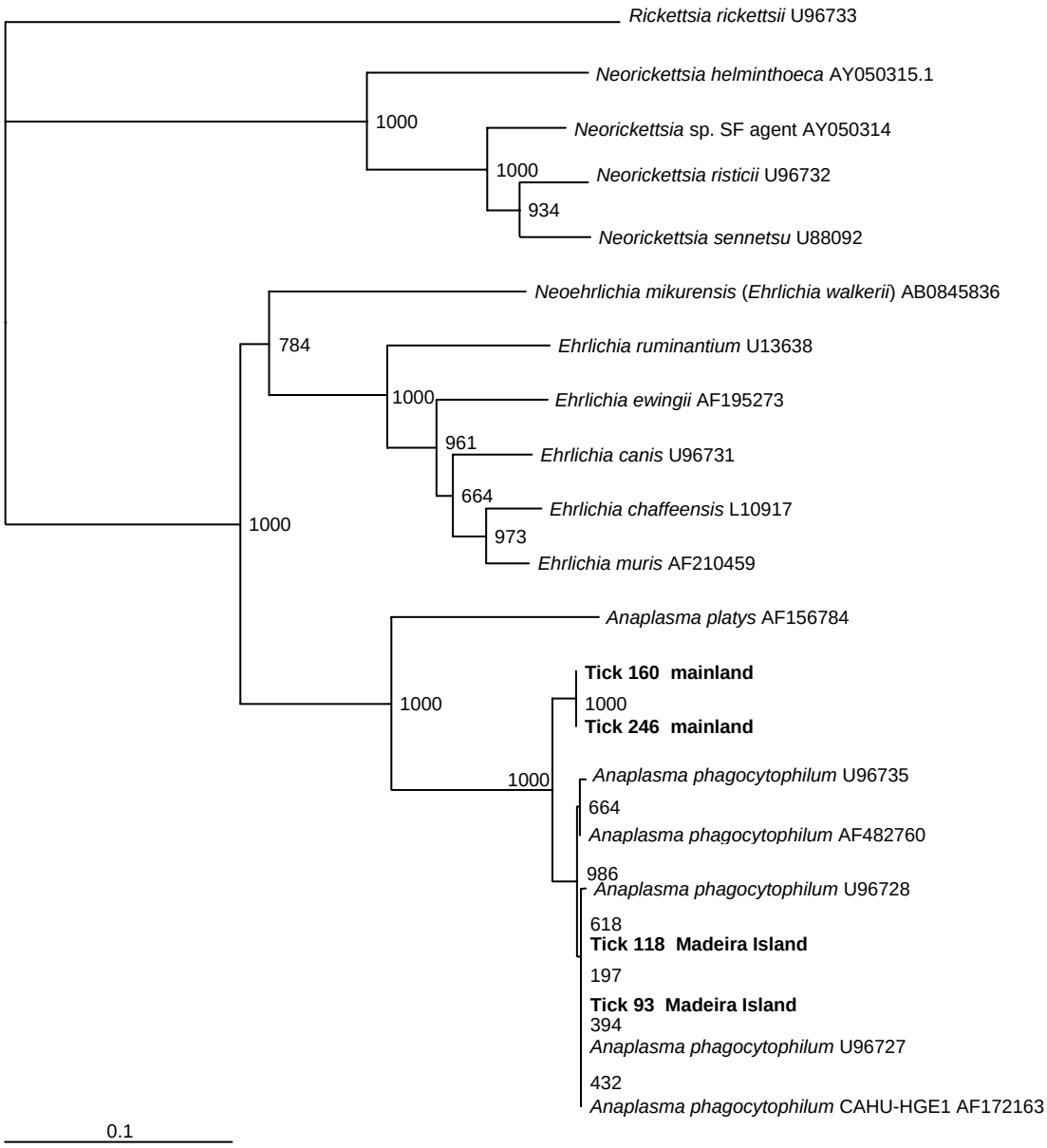
A) *msp2*



B) *rrs*


*E. ecoli* J01859
*Rickettsia rickettsii* U11021
*Neorickettsia helminthoeca* U12457
1000
*Neorickettsia sennetsu* M73219
1000
*Neorickettsia risticii* M21290
*Neoehrlichia mikurensis* (*Ehrlichia walkerii*) AF104680
453
640
*Ehrlichia ruminantium* U03776
992
*Ehrlichia muris* AB013008
679
*Ehrlichia ewingii* U96436
430
1000
*Ehrlichia canis* U26740
344
*Ehrlichia chaffeensis* M73222
*Anaplasma marginale* M60313
1000
*Anaplasma bovis* U03775
571
*Anaplasma platys* AF156784
560
*Anaplasma phagocytophilum* DQ449946
985
*Anaplasma phagocytophilum* HZ CP000235
437
*Anaplasma phagocytophilum* CAHU-HGE1 AF093788
368
*Anaplasma phagocytophilum* AJ242784
326
**Tick 118 Madeira Island**
285
*Anaplasma phagocytophilum* U02521
29
**Tick 122 Madeira Island**
20
**Tick 93 Madeira Island**
12
**Tick 246 mainland**
9
*Anaplasma phagocytophilum* AY969015
34
*Anaplasma phagocytophilum* M73224
7
**Tick 160 mainland**
0.1

C) *groESL*


*Rickettsia rickettsii* U96733
*Neorickettsia helminthoeca* AY050315.1
1000
*Neorickettsia* sp. SF agent AY050314
1000
*Neorickettsia risticii* U96732
934
*Neorickettsia sennetsu* U88092
*Neoehrlichia mikurensis* (*Ehrlichia walkerii*) AB0845836
784
*Ehrlichia ruminantium* U13638
1000
*Ehrlichia ewingii* AF195273
961
*Ehrlichia canis* U96731
664
*Ehrlichia chaffeensis* L10917
1000
973
*Ehrlichia muris* AF210459
*Anaplasma platys* AF156784
**Tick 160 mainland**
1000
1000
**Tick 246 mainland**
1000
*Anaplasma phagocytophilum* U96735
664
*Anaplasma phagocytophilum* AF482760
986
*Anaplasma phagocytophilum* U96728
618
**Tick 118 Madeira Island**
197
**Tick 93 Madeira Island**
394
*Anaplasma phagocytophilum* U96727
432
*Anaplasma phagocytophilum* CAHU-HGE1 AF172163
0.1

# CHAPTER IV

## *ANAPLASMA PHAGOCYTOPHILUM* IN NON-HUMAN VERTEBRATE HOSTS

## 4.1. DETECTION OF ANTIBODIES AGAINST *ANAPLASMA PHAGOCYTOPHILUM* IN WILD-RODENTS, PORTUGAL

SANTOS AS, AMARO F, SANTOS-SILVA MM, DE SOUSA R, MATHIAS ML, RAMALHINHO MG, NUNCIO MS, ALVES MJ, BACELLAR F; DUMLER JS

RESEARCH ARTICLE

IN SUBMISSION

# Detection of antibodies against *Anaplasma phagocytophilum* in wild-rodents, Portugal

Santos AS[1], Amaro F[1], Santos-Silva MM[1], De Sousa R[1], Mathias ML[2], Ramalhinho MG[2,3], Nuncio MS[1], Alves MJ[1], Bacellar F[1]; Dumler JS[4]

[1]Centro de Estudos de Vectores e Doenças Infecciosas, Instituto Nacional de Saúde Dr. Ricardo Jorge, Lisboa, Portugal;
[2]Centro de Biologia Ambiental, Faculdade de Ciências de Lisboa, Lisboa;
[3]Museu de História Natural, Lisboa;
[4]Division of Medical Microbiology, Department of Pathology, The Johns Hopkins University School of Medicine, Baltimore, Maryland, USA.

Corresponding author: Ana Sofia Pereira dos Santos. Centro de Estudos de Vectores e Doenças Infecciosas, Instituto Nacional de Saúde Dr. Ricardo Jorge, Edifício LEMES, Avenida Padre Cruz, 1649-016 Lisboa, Portugal;
Tel: +351 217 508 125; Fax: +351 217 508 521
e-mail: ana.santos@insa.min-saude.pt

ABSTRACT

The recent detection of *Anaplasma phagocytophilum* in Portugal stimulated further research on the agent's enzootic cycle, which usually involves rodents. Thus a total 322 rodents belonging to five species, including *Apodemus sylvaticus*, *Mus musculus*, *M. spretus*, *Rattus norvegicus* and *R. rattus*, where studied by indirect immunofluorescent assay (IFA) and/or polymerase chain reaction (PCR) for *A. phagocytophilum* exposure in four sampling areas of mainland and two areas of Madeira Island, Portugal. Overall, 3% (6/194) of *M. spretus* presented with IFA-positive results. Seropositive mice were detected in all three sampling areas of mainland where this species was captured, with prevalence of 5.2% (5/96) and 5.0% (1/20) for the *Ixodes*-areas of Arrábida and Mafra, and 1.3% (1/78) for Mértola, a difference that was not statistically significant. Regardless neither the seropositive mice nor the additional sample of 23 *M. musculus* and 5 *R. norvegicus* collected in Madeira Island showed evidence of *A. phagocytophilum* active infections when spleen and/or lung samples were tested by PCR. Either the *M. spretus* results represents residual antibodies from past *A. phagocytophilum* infections, present infections with limited bacteremia, or cross-reactions with closely related agents deserves more investigation.

RUNNING TITLE

*Anaplasma phagocytophilum* antibodies in wild-rodents, Portugal

KEYWORDS

*Anaplasma phagocytophilum*; *Apodemus sylvaticus*; *Mus musculus*; *Mus spretus; Rattus norvegicus*; *Rattus rattus*; Indirect immunofluorescent assay (IFA); Polymerase chain reaction (PCR); Portugal;

## INTRODUCTION

Rodents are important hosts for several *Ixodes* spp. ticks (Acari:Ixodidae), especially for larvae, to some extent also for nymphs, and in the case of host-specific species, for adults as well (Zeidner *et al.*, 2000; Brown *et al*., 2003, 2006). Thus, it is not surprising that rodents play a role in enzootic cycles of some *Ixodes*-borne agents, such as the human pathogens *Borrelia burgdorferi s.l*., *Babesia* spp. and tick-borne encephalitis (TBE) viruses (Labuda & Randolph, 1999; Kjemtrup & Conrad, 2000; EUCALB).

*Anaplasma phagocytophilum* [formerly *Ehrlichia phagocytophilum*, *E. equi* and human granulocytic ehrlichiosis (HGE) agent] is another example of an agent transmitted by *Ixodes* species (Dumler *et al*., 2001). This bacterium is distributed worldwide and has long been known in the veterinary field as a cause of disease in ruminants and horses, more recently also in dogs and cats, and it is additionally considered an emerging human pathogen that is increasingly being diagnosed in both North America and Europe (Dumler *et al*., 2001; Strle, 2004; Dumler, 2005; Lotric-Furland *et al*., 2006). Despite the broad-range of susceptible hosts, the occurrence of *A. phagocytophilum* variant strains with distinct pathogenicity and enzootic cycles has been suggested. Both molecular and experimental cross-infection studies has demonstrated that *A. phagocytophilum* strains associated to human, equine, and canine disease are identical or highly similar but distinct from those associated with ruminants (Chae *et al*., 2000; Pusterla *et al*., 2001; Stuen *et al*., 2003). Variant strains have been detected in ticks but the apparent lack of *A. phagocytophilum* transovarial transmission in these arthropods indicates that competent vertebrates, especially those that represent a feeding support for larvae and nymphs, play potential roles in the maintenance of agent's active cycles. Although the natural history of *A. phagocytophilum* is still unfolding, variant strains causing disease in cows and sheep are especially found in wild ruminants such as cervids (Massung *et al*., 2005, 2006). In contrast, the maintenance of variant strains involved in non-ruminant disease is believed to be dependent on tick-rodent cycles (Massung *et al*., 2003), with humans and possibly domestic animals being involved as incidental "dead-end" hosts. In the United States *Peromyscus leucopus* (white footed mouse) and *Neotoma fuscipes* (dusky footed woodrat) are competent reservoirs in Eastern and Western regions, respectively (Telford *et al*., 1996; Castro *et al.*, 2001; Foley *et al.*, 2002). Moreover, PCR-based studies show *A. phagocytophilum* DNA in several other rodent species, including *Myodes* (formerly *Clethrionomys*) *gapperi*, *Neotoma mexicana*, *Microtus orchogaster*, *Peromyscus*

*maniculatus*, *P. truei*, *Sciurus griseus*, *Spermophilus lateralis*, *Tamias minimus* and *T. striatus* in North America (Walls *et al.*, 1997; Nicholson *et al*., 1999; Zeidner *et al.*, 2000; DeNatale *et al*., 2002; Lane *et al*., 2005), *Apodemus agrarius*, *A. flavicollis, A. sylvaticus*, *Myodes* (formerly *Cletrionomys*) *glareolus, Microtus agrestis*, *M. oeconomus* and *Rattus rattus* in Europe (Liz *et al*., 2000; Brown *et al*., 2003, 2006; Christova & Gladnishka, 2005; Grzesczuk *et al*., 2006), and *Apodemus agrarius*, *A. peninsulae* and *Tamias sibiricus* in Asia (Cao *et al*., 2006).

In Portugal, *A. phagocytophilum* DNA has been detected in two *Ixodes* species, *Ixodes ricinus* collected from Madeira Island and *I. ventalloi* on the mainland (Núncio *et al*., 2000; Santos *et al*., 2004). Moreover, the Public Health importance of Portuguese strains of *A. phagocytophilum* is under study given the recent detection of active infection in a horse (Santos *et al*., in submission b) and given the presence of antibodies against this agent in *Ixodes*-exposed patients (Santos *et al*., 2006). Although no information is available regarding the natural history of *A. phagocytophilum* in Portugal, several rodents might be involved in agent's maintenance as they are known hosts for *Ixodes* species that can sustain active infection, including *Apodemus sylvaticus*, *Elyomis quercinus*, *Mus spretus*, *Rattus norvegicus* for both *I. ricinus* and *I. ventalloi*, and also *Rattus rattus* and *Sciurus vulgaris* for *I. ricinus* (Dias *et al*., 1994; MM Santos-Silva, unpublished data). Based on serological and molecular analyses of archived rodent sera and organ samples available from Center for Vector and Infectious Diseases Research, National Institute of Health Dr. Ricardo Jorge (*CEVI/INSA*), the present study investigates the potential role of Portuguese rodent species in the maintenance *A. phagocytophilum* enzootic cycles.

## MATERIAL AND METHODS

Sample collection. The rodents in this study were captured from 1998 through 2004 in three littoral areas (Arrábida, Gerês, Mafra) and one inland area (Mértola) of mainland Portugal and from 1998 through 1999 on Madeira Island (Santana, Seixal). In all the mailand littoral areas and Madeira Island the existence of *Ixodes* species was previously documented (further designated as *Ixodes*-areas). The animals were captured as part of ongoing projects to study rodent population dynamics and rodent-borne diseases by using baited Sherman and Tomahawk live-traps (H. B. Sherman Traps, Inc., Tallahassee, Florida; Tomahawk Live Traps Company, Tomahawk, Wisconsin). One trapping session was performed per season on the mainland, and only in spring

on Madeira Island, each conducted over 3 consecutive nights. Trapping and handling procedures were approved by the Institute for Conservation of Nature and Biodiversity (*ICNB*). Once in the laboratory, rodents were anesthetized with ethyl ether and biometric measurements were recorded to aid in identification to the species level. The presence of ectoparasites was investigated (no ectoparasite specimens were available for *A. phagocytophilum* testing) and whole-blood samples were collected in serum-gel microtainers (Microtainer; Becton Dickinson, New Jersey) by submandibular venous plexus puncture. Sera were recovered from blood either by gravity sedimentation or by centrifugation at 1000 x g for 10 min, and saved for serology. After euthanasia by cervical dislocation, organs were aseptically harvested, in most cases including spleen and lung samples (mainland specimens) or only lung samples (Madeira Island specimens); samples were individually cryopreserved in liquid nitrogen until use.

Serology. For detection of *A. phagocytophilum* antibodies, an indirect immunofluorescent assay (IFA) was used to screen all the available rodent sera. This included only mainland specimens, since no sera were available from Madeira Island rodents. IFA was performed according to *CEVDI/INSA* procedure using A*. phagocytophilum* Webster strain as a source of antigen. Briefly, uninfected HL-60 human promyelocytic leukaemia cells (ATCC CCL-240), at a concentration of $2x10^5$ cells/ml, were inoculated at a 3:1 ratio with *A. phagocytophilum*-infected HL-60 cells, and maintained in antibiotic-free RPMI-1640 medium (Gibco-BRL, UK), supplemented with 2 mM L-glutamine (Gibco-BRL, UK), 1% heat-inactivated fetal bovine serum (FBS; Gibco-BRL, UK) at 37ºC in 5% $CO_2$ atmosphere. When cultures reached an infection rate of 85-95%, cells were harvested by a low speed centrifugation (400 x g for 10 min) and resuspended in 0.1M phosphate buffered saline (PBS; Sigma-Aldrich, Germany) containing 2% FBS and 0.05% sodium azide. The optimal cell concentration was empirically determined by microscopic inspections of Diff-Quik (Medion Diagnostics GmbH, Germany) -stained preparations. Diluted antigen was applied to the wells of teflon-covered slides, allowed to air dry, fixed in cold acetone for 10 min and stored at -20ºC until use. For detection of *A. phagocytophilum* antibodies, sera were initially diluted at 1:40 in PBS and applied to antigen-coated wells. Serum obtained from laboratory-reared mice after *A. phagocytophilum* infection (positive control) and PBS (negative control) were included on each antigen slide. After incubation for 30 min at 37ºC in a humidified chamber, slides were washed twice in PBS for 5 min, passed through distilled water and air dried. Bound antibodies were dectected after an additional incubation of antigen wells with fluorescein isothocyanate (FITC)-labelled rabbit anti-mouse immunoglobulin G (IgG) (Dako Cytomation, Denmark) or anti-rat IgG

(Serotec, UK) in an optimal dilution of 1:40 in PBS containing 1:6000 Evans blue. The slides were washed as above, mounted with 0.3 M 1,4-diazabicyclo[2.2.2]octane (DABCO; Sigma-Aldrich, Germany) in glycerol-PBS (9:1) buffer at pH 9.0 and examined by fluorescence microscopy. Reactions were assessed for distribution, quality, morphology and intensity of fluorescent staining. Specimens were considered reactive when homogeneous fluorescence of *A. phagocytophilum* morulae or individual organisms were observed in the cytoplasm of HL-60 cells in a distribution similar to that observed using positive control sera. For the purpose of this study an IFA titer ≥40 was interpreted as seroreactive, and was subsequently serially diluted to determine the end-point titer, which was reported as the reciprocal of the highest dilution at which specific fluorescence is observed. The independence of proportions of rodent reactive sera was compared using Fisher's exact test. An alpha level of 0.05 was selected to indicate statistical significance.

Molecular analysis. Organ samples from all seropositive rodents captured on mainland Portugal and from 16% of rodents obtained from Madeira Island were tested for *A. phagocytophilum* DNA by polymerase chain reaction (PCR). Genomic DNA was extracted from available spleen (10 mg) and lung (20 mg) samples following the Qiamp Tissue Kit procedure (Qiagen GmbH, Germany). Samples from naïve laboratory-reared mice *A. phagocytophilum* were included as extraction controls. Molecular analysis was performed using nested or single tube PCR reactions target three *A. phagocytophilum* genes with several sets of primers: GE3a/GE10r followed by GE9f/GE2 for the amplification of a 546-bp sequence of *rrs* (16S rRNA gene) (Massung *et al.*, 1998); HS1/HS6 followed by HS43/HS45 for the amplification of a 442-bp sequence of the *groESL* heat-shock operon, (Sumner *et al.*, 1997); and the species-specific MSP465f/MSP980r for the amplification of a 550-bp region including the central hypervariable domain of the major surface protein-2 (*msp2* or *p44*) genes (Caspersen *et al*., 2002). Primer sequences and amplification conditions were previously described (Sumner *et al*., 1997; Massung *et al*., 1998; Caspersen *et al*., 2002). PCR was performed in a total volume of 50 μl, containing 1 μM of each primer, 2.5 U of Taq DNA polymerase and 200 μM of each deoxynucleotide triphosphate and 50 mM KCl 10 mM Tris-HCL pH 8.3, and 1.5 mM $Mg^{2+}$ (Eppendorf MasterTaq Kit, Germany), and 10 μl DNA extract (or 1 μl of amplicon in nested reactions). Water and DNA from known negative samples and *A. phagocytophilum* Webster strain genomic DNA were tested in each PCR run as controls. Prevention of cross-contamination was managed by using barrier

pipette tips and by performing PCR in a separate room from that used for DNA extraction.

## RESULTS

A total of 322 rodents belonging to five species in four genera were included in this study, comprising 30 *Apodemus sylvaticus* (wood mouse), 65 *Mus musculus* (house mouse), 194 *M. spretus* (algerian mouse), 5 *Rattus norvegicus* (brown rat) and 28 *R. rattus* (black rat). The distribution of rodent species per sampling area in mainland Portugal and Madeira Island are presented in figure 1.

The 294 rodents captured on the mainland, including 30 *A. sylvaticus*, 42 *M. musculus*, 194 *M. spretus* and 28 *R. rattus*, were initially screened by IFA for the presence of *A. phagocytophilum* antibodies. Overall, 7 mice identified as *M. spretus* were found to be seroreactive, presenting with IFA titters ranging from 80 to 320 (Table 1). The IFA-reactive *M. spretus* were detected in all three sampling areas where this species was captured, including Arrábida, Mafra and Mértola. The highest seroprevalences were observed in the *Ixodes*-areas of Arrábida and Mafra (n=5 [5.2%] and n=1 [5%], respectively), although the difference was not statistically significant when compared to Mértola (n=1 [1.3%]; $P>0.22$). Moreover, *M. spretus* were not more likely to have *A. phagocytophilum* antibodies ($P=0.09$) than the other species. Seroreactive mice were captured in all seasons: August 2002 (n=1), March 2003 (n=2), January 2004 (n=1) and April 2004 (n=1) in Arrábida; Jun 2000 (n=1) in Mafra; October 2003 (n=1) in Mértola. Analysis of proportions showed that spring was the season when seroreactive mice where more likely to be found when considering either Arrábida alone ($P=0.026$) or all *M. spretus* sampling areas together ($P=0.021$). Spleen and lung samples from all IFA-positive mice were additionally tested by PCR for the presence of *A. phagocytophilum* DNA but no positive results were detected (Table 1).

The 28 rodents captured in Madeira Island, including 23 *M. musculus* and 5 *R. norvegicus*, were tested only by PCR, since no sera samples were available at *CEVDI/INSA* for serology. PCRs performed on lung samples were negative for the three *A. phagocytophilum* genes analysed (Table 1).

## DISCUSSION

This study provides evidence for exposure of *M. spretus* to *A. phagocytophilum* or a closely related agent in Portugal. Seropositive mice were detected in all sampling areas where this species was captured, including the *Ixodes*-areas of Arrábida and Mafra, as well as Mértola. Of interest is that collection sites in Arrábida were located in the same valley and nearby the area defined as Baixa de Palmela (Setúbal District) where a previous PCR-based study detected *A. phagocytophilum* infections in *I. ventalloi* ticks (Santos *et al*., 2004). Moreover, *M. spretus* is a known vertebrate host for both *I. ventalloi* and *I. ricinus* ticks (Dias *et al*., 1994); the latter tick species is regarded as the major vector of *A. phagocytophilum* in Southern, Central and Northern Europe (Dumler *et al*., 2001; Strle, 2004). Thus these mice could be involved both in *A. phagocytophilum* primary infection cycles, promoting agent circulation between the exophylic *I. ricinus*, and in parallel cycles between endophylic species, or ticks with a more limited questing behaviour, such as *I. ventalloi*. This double involvement in *A. phagocytophilum* cycles occurs with other rodents, as with *Neotoma* sp. parasitized by *I. pacificus* and *I. spinipalpis* ticks in Colorado (Zeidner *et al.*, 2000; Burkot *et al*., 2001), and with *M. glareolus*, and *M. agrestis* parasitized by *I. ricinus* and *I. trianguliceps* ticks in United Kingdom (Bown *et al*., 2003, 2006).

In general, *M. spretus* mice can be found across the country especially in grassland, arable land, and rural gardens, selecting for humid biotypes. This mouse is usually crepuscular to nocturnal and is maximally active during spring through summer. The period from spring to early summer is also regarded as the time when most of *A. phagocytophilum* infections or reinfections occur in rodents, a seasonality that possibly results from the abundance of *Ixodes* spp. nymphs, and has been demonstrated in multicapture studies by examining PCR and/or by examining increase in specific antibody titers of rodent populations (Stafford *et al*., 1999; Castro *et al*., 2001). In our study, spring was indeed the season in which the most significant number of *A. phagocytophilum* reactive mice were detected. However, no other statistically significant difference was found in the proportion that were seropositive, even between rodent species or sampling areas, probably due to the limited number of animals studied in each location.

In areas where *A. phagocytophilum* cycles are known to occur, a proportion of rodents are simultaneously PCR and IFA-positive, varying from 20-68% for *P. leucopus* (Walls *et al*., 1997; Yeh *et al*., 1997; Stafford *et al*, 1999; Levin *et al*., 2002), 58-100% for *N. fuscipes* (Nicholson *et al*., 1999; Castro *et al*., 2001; Foley *et al*., 2002) and 20-33% for *P. truei* (Nicholson *et al*., 1999; Castro

*et al*., 2001). These findings seem to reflect either the occurrence of persistent infections or the interval during which immunity is strengthening before the elimination or suppression of bacteremia. Most of the studies regarding seasonal dynamics of *A. phagocytophilum* showed that although the majority of infections appeared to be transient, in a limited number of rodents, the agent can persist. Active infections of *A. phagocytophilum* can last at least 1 month in naturally-infected *M. glareolus* in United Kingdom (Brown *et al*., 2003), 60 days-14 months in naturally and experimentally infected *N. fuscipes* in Western US (Castro *et al*., 2001; Foley *et al*., 2002), and 1-10 months in naturally and experimentally infected *P. leucopus* (Stafford *et al*., 1999; Levin *et al*., 2002; Massung *et al*., 2004). Additionally, Levin and Fish (2000) showed that seropositive *P. leucopus* are only partially protected from reinfection. Regardless, in our case no *A. phagocytophilum* IFA-reactive *M. spretus* had an active infection, even using a cut-off criteria of 1:16-1:80 as the referenced studies (Walls *et al*., 1997; Yeh *et al*., 1997; Stafford *et al*., 1999; Castro *et al*., 2001; Nicholson *et al*., 2001; Foley *et al*., 2002; Levin *et al*., 2002). Since the range, distribution, and magnitude of antibody titers produced by most wildlife species for *A. phagocytophilum* infection are largely unknown, it is prudent to avoid setting an arbitrary cut-off. Analysis of low titers is essential, particularly when little is known and clinical signs of illness cannot be discerned (as often the case in wildlife), although the risk is false positive serological reactions that could bias seroprevalence results. As a compromise for sensitivity and specificity, all sera were screened at 1:40, a dilution bellow the cut-off value currently used in our laboratory for human serology that is broadly accepted as the minimal definition of seropositivity against *A. phagocytophilum* (Dumler *et al*., 1995). In fact, all samples that were found reactive in the initial dilution also had detectable antibody at a dilutions equal to or above 1:80. Moreover, molecular analyses were performed using PCR protocols generally considered to be highly sensitive (Sumner *et al*., 1997; Massung *et al*., 1998; Caspersen *et al*., 2002) and using organ samples that are widely accepted as adequate for detection of *A. phagocytophilum* infection in both natural and experimentally-infected rodents (Liz *et al*., 2000; Martin *et al*., 2000).

The presence of residual antibodies could result from past infections or active *A. phagocytophilum* infection with low level bacteremia that is undetectable by current methods. In fact, inefficient transient infections in mice were described in an experimental study using Ap-variant 1, a dominant *A. phagocytophilum* genotype that seems important in ruminant disease (Massung *et al*., 2003). *A. phagocytophilum* identified in Portuguese ticks seems distinct from Ap-variant 1, and at least for those detected in *I. ricinus*, similar sequences as those in non-ruminant

disease strains were observed. These data imply that infection of mice and other small mammals is likely important in natural maintenance of *A. phagocytophilum* in Portugal. Besides rodents, both *I. ricinus* and *I. ventalloi* may parasitize several other small and medium size mammalian hosts that could be potential reservoirs for *A. phagocytophilum*, including members of the orders Soricomorpha, Lagomorpha, Erinaceomorpha, and Carnivora (Dias *et al*., 1994; MM Santos-Silva unpublished data). Moreover, the potential presence of *A. phagocytophilum* ruminant strains in our country can not be excluded because no study has yet addressed this animal population. A Spanish study performed on *I. ricinus* collected from bovines identified the presence of Ap-variant 1, and this proximity is motivation for further study (Portillo *et al*., 2005). Apart from the economic relevance of *A. phagocytophilum* ruminant strains, a new and interesting epidemiological context for these strains has been recently suggested. This concept argues that *A. phagocytophilum* ruminant strains could interfer or compete with the non-ruminant strains for tick niches, perhaps influencing the incidence or prevalence of human and non-ruminant animal infections (Massung *et al*., 2002, 2003). Finally, it is also important to consider the possibility of cross-reactions with agents that share antigenic similarities with *A. phagcocytophilum*. For example the closely related *A. platys* recently detected in Portugal among dogs with active infections stimulated serologic reactions against *A. phagocytophilum* (Santos *et al*., in submission a); although, other details regarding the enzootic cycle of *A. platys* are largely unknown active infections in rodents have been already documented in other countries (Chae *et al.*, 2003; Kim *et al.*, 2006).

Rodents from the other *Ixodes*-area on Madeira Island were also studied by PCR for the presence of *A. phagocytophilum* infections, but no positive results were obtained in the 16% of the captured animals available for analysis. *R. norvegicus* and *M. musculus* were the only species captured in the two sampling areas of Santana and Seixal, despite the fact that the rodent fauna of this Island also includes *R. rattus* (Almeida, 1996). To date, no report has yet described *R. norvegicus* as a potential reservoir for *A. phagocytophilum*. In contrast, the peridomestic *M. musculus* is known to be susceptible to infection by this agent, and several inbred strains derived from this species are frequently used in experimental infections with *A. phagocytophilum* (Borjesson & Barthold, 2002). Other studies have demonstrated the persistence of *A. phagocytophilum* in Madeira Island, since infected *I. ricinus* were detected at different periods of time (Núncio *et al*., 2000; Santos *et al*., 2004). Yet whether the agent's cycles are supported by rodents or other vertebrate animals remains unknown. This Island presents a great variety of fauna that could serve as hosts for *I. ricinus*, including several bird species, bats, ferrets, goats, sheep,

cows, horses, dogs and cats (domestic and feral). The amphibian *Rana peresi* and the lizzard, *Teira dugesii* (formerly referred to as *Lacerta dugesii*) are also described (Almeida, 1996). The presence of an *A. phagocytophilum* strain closely related to human disease strains in North America (Santos *et al*., 2004, in submission c), argues for a more detailed and directed study to investigate the agent's enzootic cycles and the potential vertebrate reservoir hosts in this Portuguese Island.

ACKNOWLEDGEMENTS

This research was partially supported by the Portuguese government through the *Fundação para a Ciência e a Tecnologia* grant BD/8610/2002; Rodents capture was supported by *Instituto Nacional de Saúde Dr. Ricardo Jorge*, *Fundação para a Ciência e a Tecnologia* POCTI/ESP/39549/2001.

REFERENCES

1. ALMEIDA, V. [Ixodídeos da zona planaltica do Paul da Serra. Contribuição ao estudo da sua ecologia]. Méd Vet. 1996;46:5-22. [Article in Portuguese].

2. BORJESSON DL, BARTHOLD SW. The mouse as a model for investigation of human granulocytic ehrlichiosis: current knowledge and future directions. Comp Med. 2002;52(5):403-13.

3. BOWN KJ, BEGON M, BENNETT M, BIRTLES RJ, BURTHE S, LAMBIN X, TELFER S, WOLDEHIWET Z, OGDEN NH. Sympatric *Ixodes trianguliceps* and *Ixodes ricinus* ticks feeding on field voles (*Microtus agrestis*): potential for increased risk of *Anaplasma phagocytophilum* in the United Kingdom? Vector Borne Zoonotic Dis. 2006;6(4):404-10.

4. BOWN KJ, BEGON M, BENNETT M, WOLDEHIWET Z, OGDEN NH. Seasonal dynamics of *Anaplasma phagocytophila* in a rodent-tick (*Ixodes trianguliceps*) system, United Kingdom. Emerg Infect Dis. 2003;9(1):63-70.

5. BURKOT TR, MAUPIN GO, SCHNEIDER BS, DENATALE C, HAPP CM, RUTHERFORD JS, ZEIDNER NS. Use of a sentinel host system to study the questing behavior of *Ixodes spinipalpis* and its role in the transmission of *Borrelia bissettii*, human granulocytic ehrlichiosis, and *Babesia microti*. Am J Trop Med Hyg. 2001;65(4):293-9.

6. CASPERSEN K, PARK J-H, PATIL S, DUMLER JS. Genetic variability and stability of *Anaplasma phagocytophila* msp2 (p44). Infect Immun. 2002;70(3):1230-4.

7. CASTRO MB, NICHOLSON WL, KRAMER VL, CHILDS JE. Persistent infection in *Neotoma fuscipes* (Muridae: Sigmodontinae) with *Ehrlichia phagocytophila sensu lato*. Am J Trop Med Hyg. 2001;65(4):261-7.

8. CAO WC, ZHAN L, HE J, FOLEY JE, DE VLAS SJ, WU XM, YANG H, RICHARDUS JH, HABBEMA JD. Natural *Anaplasma phagocytophilum* infection of ticks and rodents from a forest area of Jilin Province, China. Am J Trop Med Hyg. 2006;75(4):664-8.

9. CHAE JS, FOLEY JE, DUMLER JS, MADIGAN JE. Comparison of the nucleotide sequences of 16S rRNA, 444 Ep-ank, and *groESL* heat shock operon genes in naturally occurring *Ehrlichia equi* and human granulocytic ehrlichiosis agent isolates from Northern California. J Clin Microbiol. 2000;38:1364–9.

10. CHAE JS, KIM CM, KIM EH, HUR EJ, KLEIN TA, KANG TK, LEE HC, SONG JW. Molecular epidemiological study for tick-borne disease (*Ehrlichia* and *Anaplasma* spp.) surveillance at selected US Military Training Sites/Installations in Korea. Ann NY Acad Sci. 2003;990:118-25.

11. CHRISTOVA I, GLADNISHKA T. Prevalence of infection with *Francisella tularensis*, *Borrelia burgdorferi* sensu lato and *Anaplasma phagocytophilum* in rodents from an endemic focus of tularemia in Bulgaria. Ann Agric Environ Med. 2005;12(1):149-52.

12. DENATALE CE, BURKOT TR, SCHNEIDER BS, ZEIDNER NS. Novel potential reservoirs for *Borrelia sp*. and the agent of human granulocytic ehrlichiosis in Colorado. J Wildl Dis. 2002;38(2):478-82.

13. DIAS JA, NÚNCIO MS, GONÇALVES A. [Contribuição para a elaboração de um inventário da fauna ixodoideológica (Acarina-Ixodoidea) de Portugal]. Garcia de Orta. 1994;20(1-2):49-68. [Article in Portuguese and abstract in Engish].

14. DUMLER JS. *Anaplasma* and *Ehrlichia* infection. Ann N Y Acad Sci. 2005;1063:361-73.

15. DUMLER JS, ASANOVICH KM, BAKKEN JS, RICHTER P, KIMSEY R, MADIGAN JE. Serologic cross-reactions among *Ehrlichia equi*, *Ehrlichia phagocytophila*, and human granulocytic *Ehrlichia*. J Clin Microbiol. 1995;33(5):1098–103.

16. DUMLER JS, BARBET AF, BEKKER CPJ, DASCH GA, PALMER GH, RAY SC, RIKIHISA Y, RURANGIRWA FR. Reorganization of genera in the families Rickettsiaceae and Anaplasmataceae in the order Rickettsiales: unification of some species of *Ehrlichia* with *Anaplasma*, *Cowdria* with *Ehrlichia* and *Ehrlichia* with *Neorickettsia*, description of six new species

combinations and designation of *Ehrlichia equi* and 'HGE agent' as subjective synonyms of *Ehrlichia phagocytophila*. Int J Syst Evol Microbiol. 2001;51:2145-65.

17. EUCALB- European Union Xoncerted Action on Lyme Borreliosis [updated 2006 Apr]. Available from: http://www.oeghmp.at/eucalb/diagnosis_clinical-features-ds.html.

18. FOLEY J, KRAMER V, WEBER D. Experimental infection of dusky-footed wood rats (*Neotoma fuscipes*) with *Ehrlichia phagocytophia sensu lato*. J Wildl Dis. 2002;38(1):194–8

19. GRZESZCZUK A, KARBOWIAK G, ZIARKO S, KOVALCHUK O. The root-vole *Microtus oeconomus* (Pallas, 1776): a new potential reservoir of *Anaplasma phagocytophilum*. Vector Borne Zoonotic Dis. 2006;6(3):240-3.

20. KIM CM, YI YH, YU DH, LEE MJ, CHO MR, DESAI AR, SHRINGI S, KLEIN TA, KIM HC, SONG JW, BAEK LJ, CHONG ST, O'GUINN ML, LEE JS, LEE IY, PARK JH, FOLEY J, CHAE JS. Tick-bornerickettsial pathogens in ticks and small mammals in Korea. Appl Environ Microbiol. 2006;72(9):5766-76.

21. KJEMTRUP AM, CONRAD PA. Human babesiosis: an emerging tick-borne disease. Int J Parasitol. 2000;30(12-13):1323-37.

22. LABUDA M, RANDOLPH SE. Survival strategy of tick-borne encephalitis virus: cellular basis and environmental determinants. Zentralbl Bakteriol. 1999;289(5-7):513-24.

23. LANE RS, MUN J, EISEN RJ, EISEN L. [Western gray squirrel (Rodentia: Sciuridae): a primary reservoir host of *Borrelia burgdorferi* in Californian oak woodlands?] J Med Entomol. 2005; 42(3):388-96. [Erratum in: J Med Entomol. 2005;42(4):iv].

24. LEVIN ML, FISH D. Immunity reduces reservoir host competence of *Peromyscus leucopus* for *Ehrlichia phagocytophila*. Infect Immun. 2000;68(3):1514-8.

25. LEVIN ML, NICHOLSON WL, MASSUNG RF, SUMNER JW, FISH D. Comparison of the reservoir competence of medium-sized mammals and *Peromyscus leucopus* for *Anaplasma phagocytophilum* in Connecticut. Vector Borne Zoonotic Dis. 2002;2(3):125-36.

26. LIZ JS, ANDERES L, SUMNER JW, MASSUNG RF, GERN L, RUTTI B, BROSSARD M. [PCR detection of granulocytic ehrlichiae in *Ixodes ricinus* ticks and wild small mammals in western Switzerland]. J Clin Microbiol. 2000;38(3):1002-7. [Erratum in: J Clin Microbiol 2001;39(2):828].

27. LOTRIC-FURLAN S, ROJKO T, PETROVEC M, AVSIC-ZUPANC T, STRLE F. Epidemiological, clinical and laboratory characteristics of patients with human granulocytic anaplasmosis in Slovenia. Wien Klin Wochenschr. 2006;118(21-22):708-13.

28. MARTIN ME, BUNNELL JE, DUMLER JS. Pathology, immunohistology, and cytokine

responses in early phases of human granulocytic ehrlichiosis in a murine model. J Infect Dis. 2000;181(1):374-8.

29. MASSUNG RF, COURTNEY JW, HIRATZKA SL, PITZER VE, SMITH G, DRYDEN RL. *Anaplasma phagocytophilum* in white-tailed deer. Emerg Infect Dis. 2005;11(10):1604-6.

30. MASSUNG RF, LEVIN ML, MILLER NJ, MATHER TN. Reservoir Competency of Goats for *Anaplasma phagocytophilum*. Ann NY Acad Sci. 2006;1078:476-8.

31. MASSUNG RF, MAUEL MJ, OWENS JH, ALLAN N, COURTNEY JW, STAFFORD KC 3RD, MATHER TN. Genetic variants of *Ehrlichia phagocytophila*, Rhode Island and Connecticut. Emerg Infect Dis. 2002;8(5):467-72.

32. MASSUNG RF, PRIESTLEY RA, LEVIN ML. Transmission route efficacy and kinetics of *Anaplasma phagocytophilum* infection in white-footed mouse, *Peromyscus leucopus*. Vector Borne Zoonotic Dis. 2004;4(4):310-8.

33. MASSUNG RF, PRIESTLEY RA, MILLER NJ, MATHER TN, LEVIN ML. Inability of a variant strain of *Anaplasma phagocytophilum* to infect mice. J Infect Dis. 2003;188(11):1757-63.

34. MASSUNG RF, SLATER K, OWENS JH, NICHOLSON WL, MATHER TN, SOLBERG VB, OLSON JG. Nested PCR assay for detection of granulocytic ehrlichiae. J Clin Microbiol. 1998;36:1090–5.

35. NICHOLSON WL, CASTRO MB, KRAMER VL, SUMNER JW, CHILDS JE. [Dusky-footed wood rats (*Neotoma fuscipes*) as reservoirs of granulocytic Ehrlichiae (Rickettsiales: Ehrlichieae) in northern California]. J Clin Microbiol. 1999;37(10):3323-7. [Erratum in: J Clin Microbiol. 1999;37(12):4206].

36. NÚNCIO MS, SCHOULS LM, VAN DE POL I, BACELLAR F, FILIPE A. The role of *Ixodes ricinus* as a vector of zoonotic agents in Madeira Island. In: Proceedings of the Seventh Bienal Meeting of European Tick Study Group, Oxford, United Kingdom, 2000 April 28.

37. PORTILLO A, SANTOS AS, SANTIBAÑEZ S, PÉREZ-MARTÍNEZ L, BLANCO JR, IBARRA V, OTEO JA. Detection of a non-pathogenic *Anaplasma phagocytophila* variant in *Ixodes ricinus* from the Iberian Peninsula. Ann NY Acad of Sci. 2005;1063:333-6.

38. PUSTERLA N, ANDERSON RJ, HOUSE JK, PUSTERLA JB, DEROCK E, MADIGAN JE. Susceptibility of cattle to infection with *Ehrlichia equi* and the agent of human granulocytic ehrlichiosis. J Am Vet Med Assoc. 2001;218:1160–2.

39. SANTOS AS, ALEXANDRE N, SOUSA R, NÚNCIO MS, BACELLAR F; DUMLER JS. Anaplasmataceae as etiologic agents of canine tick-borne disease in Portugal. In submission (a).

40. SANTOS AS, BACELLAR F, DUMLER JS. Human exposure to *Anaplasma phagocytophilum* in Portugal. Ann NY Acad Sci. 2006;1078:100-5.

41. SANTOS AS, GOMES J, NUNES T, FORMOSINHO P, AGRÍCOLA R, PORTAS M, BACELLAR F; DUMLER JS. Serological survey and molecular detection of *Anaplasma phagocytophilum* infection in Portuguese horses. In submission (b).

42. SANTOS AS, SANTOS-SILVA MM, ALMEIDA VC, BACELLAR F, DUMLER JS. Detection of *Anaplasma phagocytophilum* DNA in *Ixodes* ticks (Acari: Ixodidae) from Madeira Island and Setubal District, mainland Portugal. Emerg Infect Dis. 2004;10(9):1643-8.

43. SANTOS AS, SANTOS-SILVA MM, SOUSA R, BACELLAR F, DUMLER JS. PCR-based survey of *Anaplasma phagocytophilum* in Portuguese ticks (Acari: Ixodidae). In submission (c).

44. STAFFORD KC 3RD, MASSUNG RF, MAGNARELLI LA, IJDO JW, ANDERSON JF. Infection with agents of human granulocytic ehrlichiosis, Lyme disease, and babesiosis in wild white-footed mice (*Peromyscus leucopus*) in Connecticut. J Clin Microbiol. 1999;37(9):2887-92.

45. STRLE F. Human granulocytic ehrlichiosis in Europe. Int J Med Microbiol. 2004;293(Suppl 37):27-35.

46. STUEN S, BERGSTROM K, PETROVEC M, VAN DE POL I, SCHOULS LM. Differences in clinical manifestations and hematological and serological responses after experimental infection with genetic variants of *Anaplasma phagocytophilum* in sheep. Clin Diagn Lab Immunol. 2003;10(4):692-5.

47. SUMNER JW, NICHOLSON WL, MASSUNG RF. PCR amplification and comparison of nucleotide sequences from the *groESL* heat shock operon of *Ehrlichia* species. J Clin Microbiol. 1997;35:2087-92.

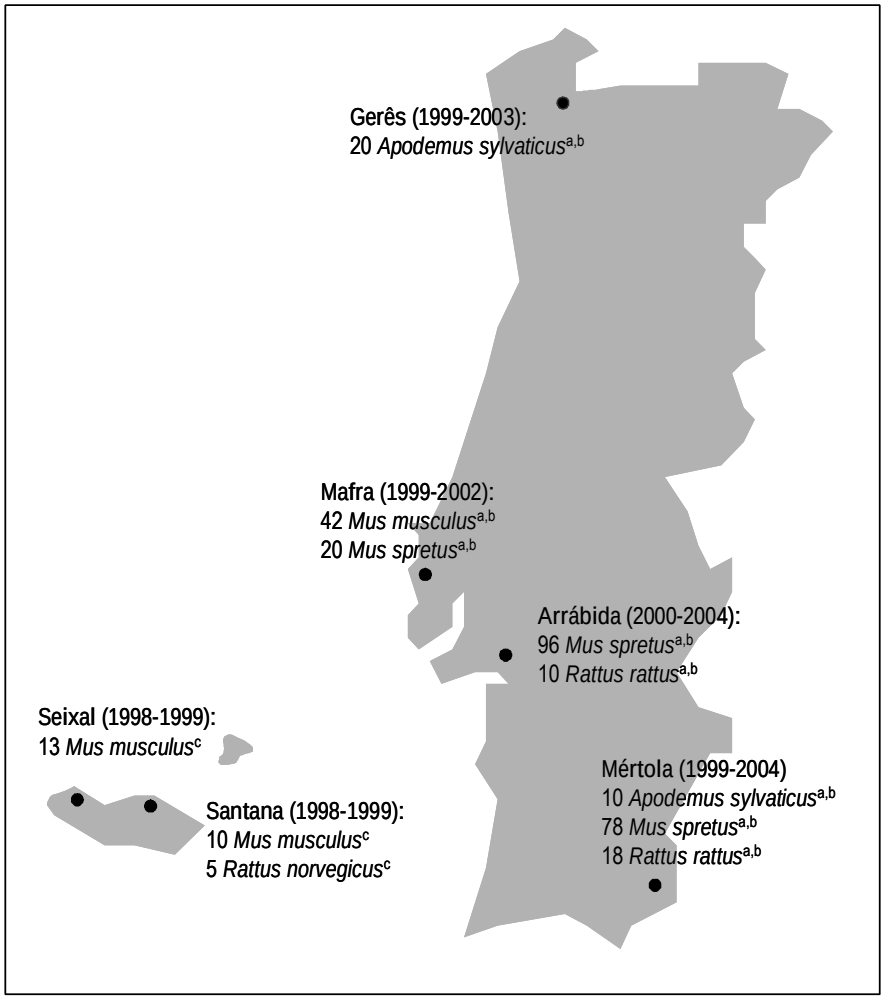
48. TELFORD S III, DAWSON J, KATAVOLOS P, WARNER C, KOLBERT C, PERSING D: Perpetuation of the agent of human granulocytic ehrlichiosis in a deer tick-rodent cycle. Proc Natl Acad Sci USA. 1996;93:6209-14.

49. WALLS JJ, GREIG B, NEITZEL DF, DUMLER JS. Natural infection of small mammal species in Minnesota with the agent of human granulocytic ehrlichiosis. J Clin Microbiol. 1997;35(4):853-5.

50. YEH MT, MATHER TN, COUGHLIN RT, GINGRICH-BAKER C, SUMNER JW, MASSUNG RF. Serologic and molecular detection of granulocytic ehrlichiosis in Rhode Island. J Clin Microbiol. 1997;35(4):944-7.

51. ZEIDNER NS, BURKOT TR, MASSUNG R, NICHOLSON WL, DOLAN MC, RUTHERFORD JS, BIGGERSTAFF BJ, MAUPIN GO. Transmission of the agent of human granulocytic ehrlichiosis by *Ixodes spinipalpis* ticks: evidence of an enzootic cycle of dual infection with *Borrelia burgdorferi* in Northern Colorado. J Infect Dis. 2000;182(2):616-9.

FIGURE 1. Total number of rodent studied by IFA and/or PCR, according to sampling area (trapping period).




[a]Studied by IFA; [b]IFA-seropositives studied by PCR (lung and spleen samples were available for testing); [c]Studied by PCR (only lung samples were available for testing

TABLE 1. *A. phagocytophilum* results obtained from mainland mice tested by both IFA and PCR and Madeira Island rodents tested by PCR.

| Sampling area | Rodent species | IFA results | | | PCR results | | | |
|---|---|---|---|---|---|---|---|---|
| | | No. tested | No. (%) positive | IFA titer | No. tested | No. (%) positive | | |
| | | | | | | *rrs* | *groESL* | *msp2* |
| Mainland Portugal: | | | | | | | | |
| Arrábida | *Mus spretus* | 96 | 5 (5.2) | 80 | 5 | - | - | - |
| Mafra | *Mus spretus* | 20 | 1 (5.0) | 320 | 1 | - | - | - |
| Mértola | *Mus spretus* | 78 | 1 (1.3) | 80 | 1 | - | - | - |
| Madeira Island: | | | | | | | | |
| Santana | *Mus musculus* | Na | Na | Na | 10 | - | - | - |
| | *Rattus norvegicus* | Na | Na | Na | 5 | - | - | - |
| Seixal | *Mus musculus* | Na | Na | Na | 13 | - | - | - |

## 4.2. SEROLOGICAL SURVEY AND MOLECULAR DETECTION OF *ANAPLASMA PHAGOCYTOPHILUM* INFECTION IN PORTUGUESE HORSES

SANTOS AS, GOMES J, NUNES T, FORMOSINHO P, AGRÍCOLA R, PORTAS M, BACELLAR F; DUMLER JS

SHORT REPORT

IN SUBMISSION

# Serological survey and molecular detection of *Anaplasma phagocytophilum* infection in Portuguese horses

Santos AS[1], Gomes J[2], Nunes T[3], Formosinho P[4], Agrícola R[5], Portas M[6], Bacellar F[1]; Dumler JS[7]

[1]Centro de Estudos de Vectores e Doenças Infecciosas, Instituto Nacional de Saúde Dr. Ricardo Jorge, Lisboa, Portugal;
[2]Laboratório Nacional de Investigação Veterinária, Lisboa;
[3]Centro de Investigação Interdisciplinar em Sanidade Animal - Faculdade de Medicina Veterinária (CIISA-FMV), Lisboa, Portugal;
[4]Laboratório Privado de Análises Clínicas, Lisboa, Portugal;
[5]Centro de Biotecnologia da Universidade dos Açores - Angra do Heroísmo, Açores, Portugal;
[6]Direcção Geral de Veterinária, Lisboa, Portugal;
[7]Division of Medical Microbiology, Department of Pathology, The Johns Hopkins University School of Medicine, Baltimore, Maryland, USA.

Corresponding author: Ana Sofia Pereira dos Santos. Centro de Estudos de Vectores e Doenças Infecciosas, Instituto Nacional de Saúde Dr. Ricardo Jorge, Edifício LEMES, Avenida Padre Cruz, 1649-016 Lisboa, Portugal;
Tel: +351 217 508 125; Fax: +351 217 508 521
e-mail: ana.santos@insa.min-saude.pt

ABSTRACT

Three hundred and two horses from 10 districts of mainland Portugal were tested for serum antibodies against *Anaplasma phagocytophilum* by indirect fluorescent antibody test (IFA). The results demonstrated a seroprevalence of 3% (9/302) with titers ranging from 80 to 640. Moreover, DNA exhibiting nucleotide sequence similarities of 98.9% (*rrs*) and 99.7% (*groESL*) to *A phagocytophilum* HZ strain were found in an IFA-positive serum sample from Faro District, Algarve region - Southern mainland. This is first definitive evidence of *A. phagocytophilum* active infection in Portuguese mammals and the zoonotic potential of this agent supports the continuing surveillance effort in the country.

RUNNING TITLE

*Anaplasma phagocytopophilum* in horses, Portugal

KEYWORDS

Tick-borne disease; *Anaplasma phagocytophilum*; Horses; Indirect Immunofluorescent Assay (IFA); Polymerase chain reaction (PCR); Portugal;

INTRODUCTION

*Anaplasma phagocytophilum* (formerly *Ehrlichia phagocytophila*, *E. equi* and HGE agent) is a worldwide, *Ixodes* tick-borne bacterium that affects several domestic animals, including ruminants, horses, dogs and cats, but is also regarded as an emerging human pathogen (Dumler *et al*., 2001). It causes a disease generically known as granulocytic anaplasmosis, formerly granulocytic ehrlichiosis, a designation that reflects the agent's tropism for polymorphonuclear leucocytes, especially neutrophils. In horses, the disease is usually mild, characterized by fever,

anorexia, depression, limb oedema, petechiae, icterus, ataxia and hematologic abnormalities, such as leukopenia, thrombocytopenia, and anemia (Madigan & Pusterla, 2000). Mortality is low, although complications from opportunistic secondary infection and injury due to ataxia can occur (Madigan & Pusterla, 2000). Equine granulocytic anaplasmosis (EGA) was initially described in 1969, in horses raised in the foothills of the Sacramento Valley, California (Gribble, 1969; Stannard *et al.*, 1969). Since then, it has been chiefly observed in California but sporadic cases of EGA have also been reported in other parts of US and Europe (Johansson *et al*., 1995; Madigan *et al*., 1996; Pusterla *et al*., 1998; Shaw *et al*., 2001; Bermann *et al*., 2002; Von Loewenich *et al*., 2003; Alberti *et al*., 2005).

The geographic distribution of EGA, as well as other non-ruminant cases of granulocytic anaplasmosis, commonly overlaps those regions where human infection occurs. In fact, several studies demonstrate that *A. phagocytophilum* strains able to cause equine, canine or human disease are genetically identical or highly similar (Johansson *et al*., 1995; Madigan *et al*., 1996; Pusterla *et al*., 1998; Bullock *et al*., 2000; Chae *et al*., 2000; Von Loewenich *et al*., 2003). This relation has also been suggested by experimental cross-infections (Barlough *et al*., 1995; Madigan *et al*., 1995; Pusterla *et al*., 2001). Thus, the study of *A. phagocytophilum* infections in horses or other non-ruminants is important not only for veterinary management, but also to predict human disease risk. In Portugal *A. phagocytophilum* is present in *Ixodes ricinus* and *I. ventalloi* ticks, but its Public Health potential is still incompletely appreciated (Santos *et al*., 2004). Using serological and molecular surveys, we studied the possibility of *A. phagocytophilum* infection in horses as part of an integrated effort to define the medical importance of this agent in Portugal.

## MATERIAL AND METHODS

Sera used in this study were obtained between 2002 and 2006 in collaboration with several Institutions, including *Serviço Nacional Coudélico* (now, *Fundação Alter-Real*), *Faculdade de Medicina Veterinária* and *Instituto Nacional de Investigação Veterinária*. The samples were collected either for routine evaluation or for serosurveillance of infectious diseases such as babesiosis, brucellosis, leptospirosis, herpes virus, contagious metritis, viral arteritis and West Nile virus. Clinical histories were not obtained, although limited information was available for some

animals. Sera were frozen at - 20ºC until assayed.

For detection of antibodies against *A. phagocytophilum*, an indirect immunofluorescent assay (IFA) was performed as previously described (Santos *et al*., in submission). Briefly, sera were diluted at 1:40 and incubated on antigen slides prepared with *A. phagocytophilum* Webster strain-infected HL-60 cells. A second incubation with fluorescein isothiocyanate (FITC)-labelled rabbit anti-horse immunoglobulin G (Sigma-Aldrich, Germany), was used to identify bound antibodies resulting in homogeneous fluorescence of *A. phagocytophilum* morulae. All reactive samples were serial diluted to determine the end-point titer, which was expressed as the reciprocal of the serum dilution. An IFA titer ≥80 was interpreted as positive. Unvariate statistical analysis for verification of the association between infection, gender, origin, and age class was performed using Fisher's exact test. Statistical analysis was performed using SAS version 9.1 (SAS Institute, Inc., 2002-2003, Cary, NC). An alpha level of 0.05 was selected to indicate statistical significance.

All IFA-positive samples were also tested for the presence of *A. phagocytophilum* DNA by polymerase chain reaction (PCR). Genomic DNA was extracted from 200 µl of horse serum using the Qiamp Blood Kit procedure (Qiagen GmbH, Germany). A known negative sample was included in every group of three sera extractions as quality control. Nested or single tube PCR reactions targeting *rrs* (16S rRNA gene), *groESL*, and *msp2* (*p44*) genes were performed as previously described (Sumner *et al*., 1997; Massung *et al*., 1998; Caspersen *et al*., 2002), in a total volume of 50 µl, containing 1 µM of each primer, 2.5 U of Taq DNA polymerase, 200 µM of each deoxynucleotide triphosphate, 50 mM KCl 10 mM Tris-HCL pH 8.3, 1.5 mM $Mg^{2+}$ (Eppendorf MasterTaq Kit, Germany), and 10 µl DNA extract (or 1 µl of amplicon in nested PCR reactions). In each run, in addition to known negatives (three extraction controls and water), known positive samples were used as controls. Prevention of cross-contamination was managed by using pipette tips with filter barrier, and performing PCR in a three separate room (extraction, master mix preparation, and amplification/electrophoresis). DNA amplicons from positive samples were purified (Jetquick PCR Purification Kit; Genomed GmbH, Germany) and sequenced using an ABI automated sequencer (Applied Biosystems). After manual review and editing, sequence homology searches were performed using BLASTn.

Nucleotide sequence accession numbers: Partial gene sequences generated for *rrs* and *groESL* were deposited in GenBank under the accession numbers EF693890, and EF693889, respectively.

## RESULTS

A total of 302 serum samples from 10 districts of Mainland Portugal were studied by IFA (Figure 1). From the limited information provided, horse age and breed were available for 38.4% and 30.8% of the cases, respectively. Individuals represented at least 6 different breeds, including Arabian (n=3), Garrano (n=33), Lusitano (n=47), Quarter (n=1), Sorraia (n=3) and Thoroughbred horses (n=6). Antibodies against *A. phagocytophilum* were detected in 3% (9/302) of sera with titers ranging from 80 to 640 (Table 1). No association was found between *A. phagocytophilum* seroprevalence and the origin of the horses. There was no consistent pattern of seropositivity among age groups or when gender was examined (Table 2). Molecular analysis of IFA-positive sera showed the presence of *A. phagocytophilum* DNA in 1 mare (Seara) from Faro District. The nucleotide sequences obtained exhibited a high degree of similarity with the human *A. phagocytophilum* HZ strain (CP000235), with values of 98.9% (459/464) and 99.7% (466/467) for partial sequences of both *rrs* and *groEL*, respectively.

## DISCUSSION

Indirect immunofluorescent antibody test (IFA) is the gold standard for routine *A. phagocytophilum* diagnosis and has been also widely applied in seroprevalence studies. Here, antibodies against *A. phagocytophilum* or another antigenically similar agent were detected in 3% of the horses studied, which is in accordance with the wide range of seroprevalence (0-17.6%) reported in other countries (Madigan *et al*., 1990; Bullock *et al*., 2000; Magnarelli *et al*., 2000; Engevall *et al*., 2001; Teglas *et al*., 2005; Amusatequi *et al*., 2006; Levi *et al*., 2006). Nevertheless, our seroprevalence is somewhat lower than that obtained in other parts of Europe (Engevall *et al*., 2001; Amusatequi *et al*., 2006), but is similar to that reported from *A. phagocytophilum* non-endemic regions in the US (Madigan *et al*., 1990; Bullock *et al*., 2000).

In Europe, *Ixodes ricinus* has long been known as a competent vector for *A. phagocytophilum* (Macleod & Gordon, 1933). This tick species present in Portugal a patchy distribution mainly influenced by climate (temperature, humidity and precipitation), landscape fragmentation, and landscape composition (open areas, mixed and deciduous forests) (Baptista,

2006). Although, it has been reported from all districts of mainland Portugal (Caeiro, 1999), the highest prevalence is registered in the littoral of the country (Baptista, 2006). An example of an *I. ricinus*-area is Gerês (in Braga District, Northern litoral) (Caeiro, 1999), from where the 33 Garrano horses studied in this work came from. These are feral horses, traditionally raised freely in the hills; thus, contact with *I. ricinus* is highly likely. Nevertheless, only *Rhipihicephalus bursa* and *Hyalomma marginatum* were found parasitizing these horses at the time of blood collection (data not shown) and none of the animals had *A. phagocytophilum* antibodies. Whether the serological results indicate absence of *A. phagocytophilum* in this region, the inability of any *A. phagocytophilum* variant strains present in *I. ricinus* in this region to establish infection in horses, or simply limited contact between horses and tick species linked to the agent´s transmission is a matter deserving further investigation.

An important limitation of IFA testing is the potential for false positive reactions. Several studies demonstrate *A. phagocytophilum* cross-reactivity with phylogenetically-related agents, such as *A. marginale*, *A. platys*, *Ehrlichia chaffeensis* and *E. canis* (Comer *et al*., 1999; Hinokuma *et al*., 2001; Waner *et al*., 2001; Dreher *et al*., 2005). Although these microorganisms are not known to infect horses, cross-reactions with heat-shock proteins or surface proteins from other non-relatedagents should not be ruled out. In fact, one horse from Braga District (named Shuma) that presented an IFA titer of 320 against *A. phagocytophilum* also had positive serologic results for both *Leptospira grippotyphosa* and equine rhinopneumonitis EHV 1 and EHV 4.

Another issue of serology is the difficulty in differentiating active infection from residual antibodies when only one serum sample is available. Follow-up studies on naturally infected horses have shown the presence of high antibody titers detectable by IFA several months after infection (Van Andel *et al*., 1998; Artursson *et al*., 1999). To overcome these limitations, serology is occasionally coupled with molecular testing to detect active *A. phagocytophilum* infections (Bullock *et al*., 2000; Teglas *et al*., 2005). In this study, the molecular analysis of all IFA-positive sera revealed *A. phagocytophilum* DNA in one animal from Faro District (named Seara). Confirmation of active *A. phagocytophilum* infection was achieved only by nested PCR, but using 2 separate assays that targeted *rrs* and *groESL* that are much more likely to reflect infection than amplicon contamination. Nested reactions are regarded as highly sensitive and are able to detect very small amounts of DNA, even in serum (Massung *et al.*, 1998). However, it is important to note that negative results obtained from the other IFA-positive horses could be falsely negative because of the inavailability of more reliable samples for molecular testing, such as whole-blood or buffy coat.

The results presented herein are the first definitive evidence of active *A. phagocytophilum* infection in Portuguese mammals, but due to inavailability of complete clinical records, a relationship between infection and disease could not be effectively analyzed. The partial nucleotide sequences of both *rrs* and *groESL* obtained from 1 horse were highly similar to those of *A. phagocytophilum* HZ strain, an isolate obtained from a New York human patient in 1995 (Rikihisa *et al*., 1997; Hottop *et al*., 2006); thus, the possibility of human infections by this agent in Portugal cannot be excluded. Algarve is an international tourist destination which receives visitors and their pets from all over the world. In addition, Algarve is in the migratory route of several bird species which could promote the introduction or spread of ticks and tick-borne pathogens. These data coupled with the zoonotic potential of *A. phagocytophilum* supports a countinuos surveillance for *A. phagocytophilum* and granulocytic anaplasmosis in Portugal.

ACKNOWLEDGEMENTS

This research was partially supported by the Portuguese government through the *Fundação para a Ciência e a Tecnologia* grant BD/8610/2002. A special thank to veterinarians and all persons from the institutions that have directly participated in the study, including the *Associação de Criadores de Equinos de Raça Garrana* and to the staff of the *Unidade Laboratorial de Utilização Comum*, *Instituto Nacional de Saude Dr. Ricardo Jorge*, for their valuable assistance with sequencing.

REFERENCES:

1. ALBERTI A, ZOBBA R, CHESSA B, ADDIS MF, SPARAGANO O, PINNA PARPAGLIA ML, CUBEDDU T, PINTORI G, PITTAU M. Equine and canine *Anaplasma phagocytophilum* strains isolated on the island of Sardinia (Italy) are phylogenetically related to pathogenic strains from the United States. Appl Environ Microbiol. 2005;71(10):6418-22.

2. AMUSATEGUI I, SAINZ A, TESOURO MA. Serological evaluation of *Anaplasma phagocytophilum* infection in livestock in northwestern Spain. Ann NY Acad Sci. 2006;1078:487-90.

ARTURSSON K, GUNNARSSON A, WIKSTROM UB, ENGVALL EO. A serological and clinical follow-up in horses with confirmed equine granulocytic ehrlichiosis. Equine Vet J.

1999;31(6):473-7.

3. BAPTISTA S. [Lyme borreliosis in Portugal. Study on vector(s), agent(s) and risk factors]. Tese de Doutoramento, Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa, Lisboa, 2006. [PhD Thesis in English].

4. BARLOUGH JE, MADIGAN JE, DEROCK E, DUMLER JS, BAKKEN JS. Protection against *Ehrlichia equi* is conferred by prior infection with the human granulocytotropic ehrlichia (HGE agent). J Clin Microbiol. 1995;33(12):3333-4.

5. BERMANN F, DAVOUST B, FOURNIER PE, BRISOU-LAPOINTE AV, BROUQUI P. *Ehrlichia equi* (*Anaplasma phagocytophila*) infection in an adult horse in France. Vet Rec. 2002;150(25):787-8.

6. BULLOCK PM, AMES TR, ROBINSON RA, GREIG B, MELLENCAMP MA, DUMLER JS. *Ehrlichia equi* infection of horses from Minnesota and Wisconsin: detection of seroconversion and acute disease investigation. J Vet Intern Med. 2000;14(3):252-7.

7. CAEIRO V. General review of tick species present in Portugal. Parassitologia. 1999;41(Suppl 1):11-5.

8. CASPERSEN K, PARK J-H, PATIL S, DUMLER JS. Genetic variability and stability of *Anaplasma phagocytophila msp2* (*p44*). Infect Immun. 2002;70(3):1230-4.

9. CHAE JS, FOLEY JE, DUMLER JS, MADIGAN JE. Comparison of the nucleotide sequences of 16S rRNA, 444 Ep-ank, and *groESL* heat shock operon genes in naturally occurring *Ehrlichia equi* and human granulocytic ehrlichiosis agent isolates from Northern California. J Clin Microbiol. 2000;38:1364–9.

10. COMER JA, NICHOLSON WL, OLSON JG, CHILDS JE. Serologic testing for human granulocytic ehrlichiosis at a National Referral Center. J Clin Microbiol. 1999;37(3):558-64.

11. DREHER UM, DE LA FUENTE J, HOFMANN-LEHMANN R, MELI ML, PUSTERLA N, KOCAN KM, WOLDEHIWET Z, BRAUN U, REGULA G, STAERK KD, LUTZ H. Serologic cross-reactivity between *Anaplasma marginale* and *Anaplasma phagocytophilum*. Clin Diagn Lab Immunol. 2005;12(10):1177-83.

12. DUMLER JS, BARBET AF, BEKKER CPJ, DASCH GA, PALMER GH, RAY SC, RIKIHISA Y, RURANGIRWA FR. Reorganization of genera in the families Rickettsiaceae and Anaplasmataceae in the order Rickettsiales: unification of some species of *Ehrlichia* with *Anaplasma*, *Cowdria* with *Ehrlichia* and *Ehrlichia* with *Neorickettsia*, description of six new species

combinations and designation of *Ehrlichia equi* and 'HGE agent' as subjective synonyms of *Ehrlichia phagocytophila*. Int J Syst Evol Microbiol. 2001;51:2145-65.

13. EGENVALL A, FRANZEN P, GUNNARSSON A, ENGVALL EO, VAGSHOLM I, WIKSTROM UB, ARTURSSON K. Cross-sectional study of the seroprevalence to *Borrelia burgdorferi sensu lato* and granulocytic *Ehrlichia* spp. and demographic, clinical and tick-exposure factors in Swedish horses. Prev Vet Med. 2001;49(3-4):191-208.

14. GRIBBLE DH. Equine ehrlichiosis. J Am Vet Med Assoc. 1969;155:462-9.

15. HOTOPP JC, LIN M, MADUPU R, CRABTREE J, ANGIUOLI SV, EISEN J, SESHADRI R, REN Q, WU M, UTTERBACK TR, SMITH S, LEWIS M, KHOURI H, ZHANG C, NIU H, LIN Q, OHASHI N, ZHI N, NELSON W, BRINKAC LM, DODSON RJ, ROSOVITZ MJ, SUNDARAM J, DAUGHERTY SC, DAVIDSEN T, DURKIN AS, GWINN M, HAFT DH, SELENGUT JD, SULLIVAN SA, ZAFAR N, ZHOU L, BENAHMED F, FORBERGER H, HALPIN R, MULLIGAN S, ROBINSON J, WHITE O, RIKIHISA Y, TETTELIN H. Comparative genomics of emerging human ehrlichiosis agents. PLoS Genet. 2006;2(2):e21.

16. INOKUMA H, OHNO K, ONISHI T, RAOULT D, BROUQUI P. Detection of ehrlichial infection by PCR in dogs from Yamaguchi and Okinawa Prefectures, Japan. J Vet Med Sci. 2001;63(7):815-7.

17. JOHANSSON KE, PETTERSSON B, UHLEN M, GUNNARSSON A, MALMQVIST M, OLSSON E. Identification of the causative agent of granulocytic ehrlichiosis in Swedish dogs and horses by direct solid phase sequencing of PCR products from the 16S rRNA gene. Res Vet Sci. 1995;58(2):109-12.

18. LEVI O, WANER T, BANETH G, KEYSARY A, BRUCHIM Y, SILVERMAN J, HARRUS S. Seroprevalence of *Anaplasma phagocytophilum* among healthy dogs and horses in Israel. J Vet Med B Infect Dis Vet Public Health. 2006;53(2):78-80.

19. MACLEOD J, GORDON WS. Studies on tick-borne fever in sheep I: transmission by the tick *Ixodes ricinus* and descripition of the disease produced. Parasitology. 1933;25:273-83.

20. MADIGAN JE, PUSTERLA N. Ehrlichial diseases. Vet Clin North Am Equine Pract. 2000;16(3):487-99.

21. MADIGAN JE, BARLOUGH JE, DUMLER JS, SCHANKMAN NS, DEROCK E. Equine granulocytic ehrlichiosis in Connecticut caused by an agent resembling the human granulocytotropic ehrlichia. J Clin Microbiol. 1996;34(2):434-5.

22. MADIGAN JE, HIETALA S, CHALMERS S, DEROCK E. Seroepidemiologic survey of antibodies to *Ehrlichia equi* in horses of northern California. J Am Vet Med Assoc. 1990;196(12):1962-4.

23. MADIGAN JE, RICHTER PJ JR, KIMSEY RB, BARLOUGH JE, BAKKEN JS, DUMLER JS. Transmission and passage in horses of the agent of human granulocytic ehrlichiosis. J Infect Dis. 1995;172(4):1141-4.

24. MAGNARELLI LA, IJDO JW, VAN ANDEL AE, WU C, PADULA SJ, FIKRIG E. Serologic confirmation of *Ehrlichia equi* and *Borrelia burgdorferi* infections in horses from the northeastern United States. J Am Vet Med Assoc. 2000;217(7):1045-50.

25. MASSUNG RF, SLATER K, OWENS JH, NICHOLSON WL, MATHER TN, SOLBERG VB, OLSON JG. Nested PCR assay for detection of granulocytic ehrlichiae. J Clin Microbiol. 1998;36:1090–5.

26. PUSTERLA N, ANDERSON RJ, HOUSE JK, PUSTERLA JB, DEROCK E, MADIGAN JE. Susceptibility of cattle to infection with *Ehrlichia equi* and the agent of human granulocytic ehrlichiosis. J Am Vet Med Assoc. 2001;218:1160–2.

27. PUSTERLA N, HUDER JB, FEIGE K, LUTZ H. Identification of a granulocytic *Ehrlichia* strain isolated from a horse in Switzerland and comparison with other rickettsiae of the *Ehrlichia phagocytophila* genogroup. J Clin Microbiol. 1998;36(7):2035-7.

28. RIKIHISA Y, ZHI N, WORMSER GP, WEN B, HOROWITZ HW, HECHEMY KE. Ultrastructural and antigenic characterization of a granulocytic ehrlichiosis agent directly isolated and stably cultivated from a patient in New York State. J Infect Dis. 1997;175:210-3.

29. SANTOS AS, AMARO F, SANTOS-SILVA M, SOUSA R, MATHIAS ML, RAMALHINHO MG, NUNCIO MS, ALVES MJ, BACELLAR F; DUMLER JS. Detection of antibodies against *Anaplasma phagocytophilum* in wild-rodents, Portugal. In submission.

30. SANTOS AS, SANTOS-SILVA MM, ALMEIDA VC, BACELLAR F, DUMLER JS. Detection of *Anaplasma phagocytophilum* DNA in *Ixodes* ticks (Acari: Ixodidae) from Madeira Island and Setubal District, mainland Portugal. Emerg Infect Dis. 2004;10(9):1643-8.

31. SHAW S, KENNY M, TAYLOR F, DAY M, BIRTLES R, PEARCE C. Equine granulocytic ehrlichiosis in the UK. Vet Rec. 2001;149(4):127-8.

32. STANNARD AA, GRIBBLE DH, SMITH RS. Equine ehrlichiosis. A disease with similarities to tick-borne fever and bovine petechial fever. Vet Rec. 1969;84:149-50.

33. SUMNER JW, NICHOLSON WL, MASSUNG RF. PCR amplification and comparison of nucleotide sequences from the *groESL* heat shock operon of *Ehrlichia* species. J Clin Microbiol. 1997;35:2087-92.

34. TEGLAS M, MATERN E, LEIN S, FOLEY P, MAHAN SM, FOLEY J. Ticks and tick-borne disease in Guatemalan cattle and horses. Vet Parasitol. 2005;131(1-2):119-27.

35. VAN ANDEL AE, MAGNARELLI LA, HEIMER R, WILSON ML. Development and duration of antibody response against *Ehrlichia equi* in horses. J Am Vet Med Assoc. 1998;212(12):1910-4.

36. VON LOEWENICH FD, STUMPF G, BAUMGARTEN BU, ROLLINGHOFF M, DUMLER JS, BOGDAN C. A case of equine granulocytic ehrlichiosis provides molecular evidence for the presence of pathogenic *Anaplasma phagocytophilum* (HGE agent) in Germany. Eur J Clin Microbiol Infect Dis. 2003;22(5):303-5.

37. WANER T, HARRUS S, JONGEJAN F, BARK H, KEYSARY A, CORNELISSEN AW. Significance of serological testing for ehrlichial diseases in dogs with special emphasis on the diagnosis of canine monocytic ehrlichiosis caused by *Ehrlichia canis*. Vet Parasitol. 2001;95(1):1-15.


FIGURE 1. Number of *A. phagocytophilum* seropositive cases per total horses studied in each district (origin information was not available for nine animals).

TABLE 1. *A. phagocytophilum* results obtained from mainland mice tested by both IFA and PCR and Madeira Island rodents tested by PCR.

| Sampling area | Rodent species | IFA results | | | PCR results | | | |
|---|---|---|---|---|---|---|---|---|
| | | No. tested | No. (%) positive | IFA titer | No. tested | No. (%) positive | | |
| | | | | | | *rrs* | *groESL* | *msp2* |
| Mainland Portugal: | | | | | | | | |
| Arrábida | *Mus spretus* | 96 | 5 (5.2) | 80 | 5 | - | - | - |
| Mafra | *Mus spretus* | 20 | 1 (5.0) | 320 | 1 | - | - | - |
| Mértola | *Mus spretus* | 78 | 1 (1.3) | 80 | 1 | - | - | - |
| Madeira Island: | | | | | | | | |
| Santana | *Mus musculus* | Na | Na | Na | 10 | - | - | - |
| | *Rattus norvegicus* | Na | Na | Na | 5 | - | - | - |
| Seixal | *Mus musculus* | Na | Na | Na | 13 | - | - | - |

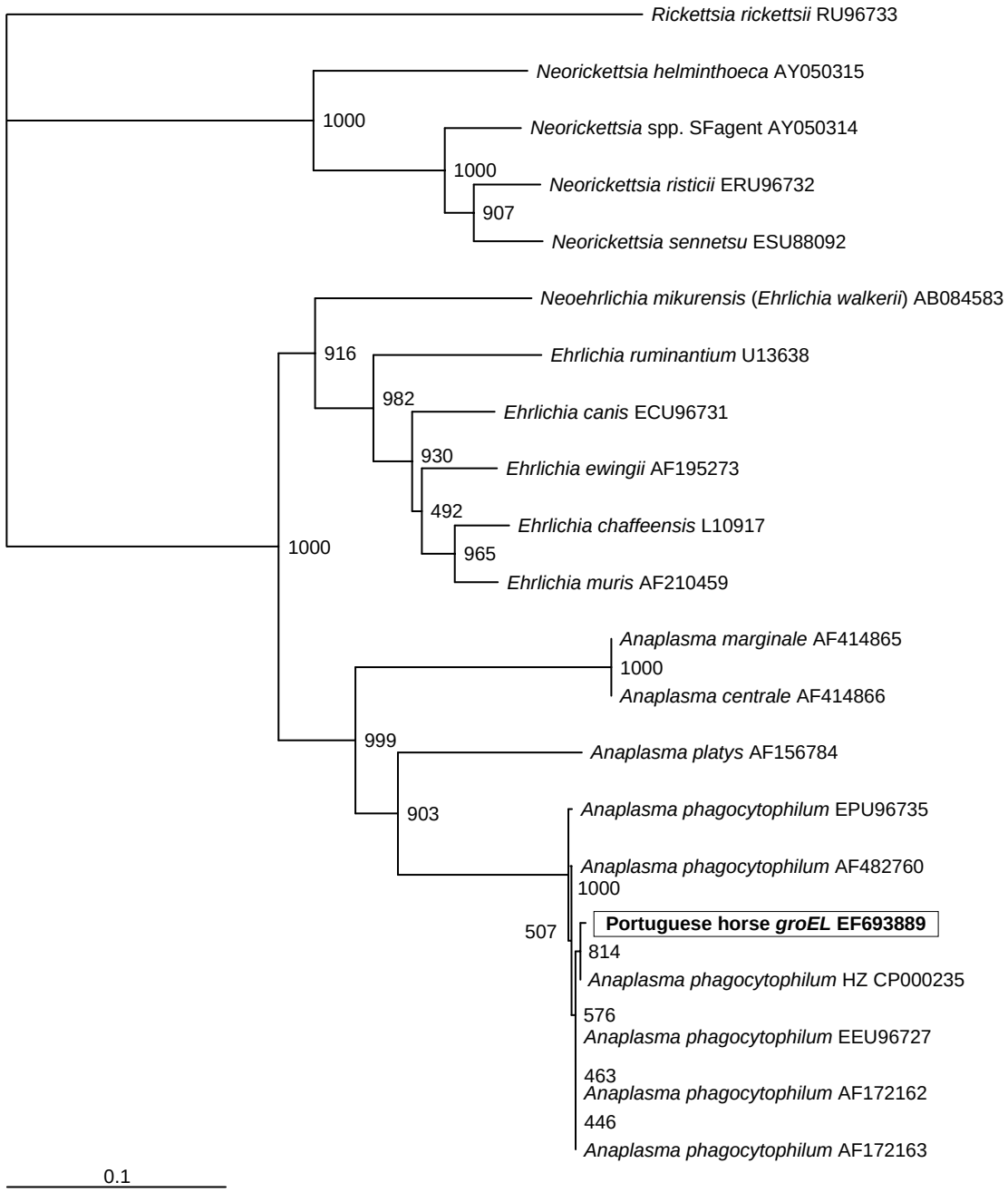
TABLE 2. Univariate statistical analysis of *A. phagocytophilum* seropositive horses according to age, gender and origin.

| Variable | No of tested horses† | No of seropositive (%) | *p value* |
|---|---|---|---|
| Age (years) | | | |
| 0-4 | 6 | 0 | 0.14 |
| 5-9 | 42 | 4 (9.5) | |
| >9 | 68 | 1(1.5) | |
| Gender | | | |
| Male | 80 | 4(5.0) | 1 |
| Female | 81 | 5(6.2) | |
| Origin | | | |
| Bragança | 37 | 1(2.7) | 0.64 |
| Beja | 5 | 0 | |
| Coimbra | 1 | 0 | |
| Évora | 15 | 0 | |
| Faro | 103 | 4(3.9) | |
| Lisboa | 24 | 1(4.2) | |
| Portalegre | 9 | 1(11.1) | |
| Porto | 3 | 0 | |
| Santarém | 74 | 1(1.4) | |
| Setúbal | 22 | 1(4.5) | |

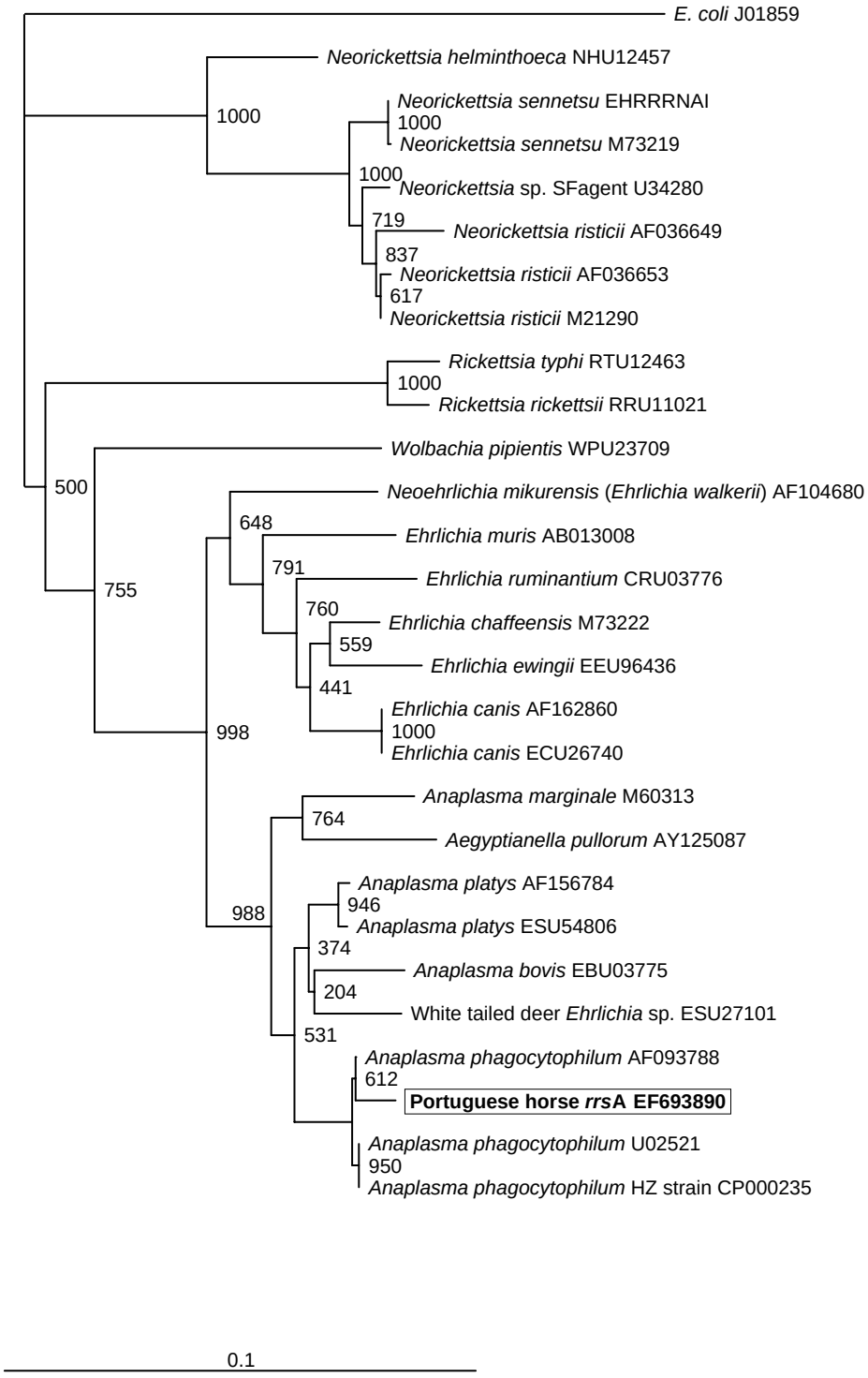
†includes only animals with available information.

FIGURE 2. Phylogenetic relationships between *A. phagocytophilum* sequences detected in this study and other Anaplasmataceae based on partial nucleotide sequences of the *groESL* (A) and *rrs (B)*. The scale bar represents substitutions per 1,000 base pairs; numbers at nodes represent bootstrap values out of 1,000 iterations. The nucleotide sequences of the index horse are shown in a box; accession numbers for each sequence are also included after the genus and species designations.

A) *groESL*



B) *rrs*


*E. coli* J01859
*Neorickettsia helminthoeca* NHU12457
1000
*Neorickettsia sennetsu* EHRRRNAI
1000
*Neorickettsia sennetsu* M73219
1000
*Neorickettsia* sp. SFagent U34280
719
*Neorickettsia risticii* AF036649
837
*Neorickettsia risticii* AF036653
617
*Neorickettsia risticii* M21290
*Rickettsia typhi* RTU12463
1000
*Rickettsia rickettsii* RRU11021
*Wolbachia pipientis* WPU23709
500
*Neoehrlichia mikurensis* (*Ehrlichia walkerii*) AF104680
648
*Ehrlichia muris* AB013008
791
*Ehrlichia ruminantium* CRU03776
755
760
*Ehrlichia chaffeensis* M73222
559
*Ehrlichia ewingii* EEU96436
441
*Ehrlichia canis* AF162860
998
1000
*Ehrlichia canis* ECU26740
*Anaplasma marginale* M60313
764
*Aegyptianella pullorum* AY125087
*Anaplasma platys* AF156784
946
988
*Anaplasma platys* ESU54806
374
*Anaplasma bovis* EBU03775
204
White tailed deer *Ehrlichia* sp. ESU27101
531
*Anaplasma phagocytophilum* AF093788
612
**Portuguese horse *rrs*A EF693890**
*Anaplasma phagocytophilum* U02521
950
*Anaplasma phagocytophilum* HZ strain CP000235
0.1

## 4.3. ANAPLASMATACEAE AS ETIOLOGIC AGENTS OF CANINE TICK-BORNE DISEASE IN PORTUGAL

SANTOS AS, ALEXANDRE N, DE SOUSA R, NÚNCIO MS, BACELLAR F; DUMLER JS

RESEARCH ARTICLE

IN SUBMISSION

# Anaplasmataceae as etiologic agents of canine tick-borne disease in Portugal

Ana Sofia Santos[1], Nuno Alexandre[2], Rita Sousa[1], Maria Sofia Núncio[1], Fátima Bacellar[1]; J. Stephen Dumler[3]

[1]Centro de Estudos de Vectores e Doenças Infecciosas, Instituto Nacional de Saúde Dr. Ricardo Jorge, Lisboa, Portugal;
[2]Departamento de Zootecnia, Universidade de Évora, Pólo da Mitra, Évora;
[3]Division of Medical Microbiology, Department of Pathology, The Johns Hopkins University School of Medicine, Baltimore, Maryland, USA.

Corresponding author: Ana Sofia Pereira dos Santos. Centro de Estudos de Vectores e Doenças Infecciosas, Instituto Nacional de Saúde Dr. Ricardo Jorge, Edifício LEMES, Avenida Padre Cruz, 1649-016 Lisboa, Portugal;
Tel: +351 217 508 125; Fax: +351 217 508 521
e-mail: ana.santos@insa.min-saude.pt

ABSTRACT

Fifty five dogs with clinical signs of suspected tick-borne disease were tested by both immunofluorescence assay (IFA) and polymerase chain reaction (PCR) for *A. phagocytophilum*. Overall, 30 (54.5%) dogs presented with IFA positive results, 5 of which fulfilled the serologic criteria for active infection presenting either seroconversion or four-fold increase in antibody titers. PCR analysis failed to detect active *A. phagocytophilum* infections, but *A. platys* DNA was found in 2 seropositive and 3 seronegative animals. This provides the first definitive evidence of *A. platys* infection in Portuguese dogs and emphasizes the importance of considering infectious cyclic thrombocytopenia in the differential diagnosis of canine tick-borne disease in Portugal.

RUNNING TITLE

Anaplasmataceae infections in dogs, Portugal

KEYWORDS

Tick-borne disease; dogs; *Anaplasma phagocytophilum*; *Anaplasma platys*; IFA; PCR; Portugal.

## INTRODUCTION

Five members of the Family Anaplasmataceae are currently implicated in canine tick-borne disease, including *Anaplasma phagocytophilum*, *A. platys*, *Ehrlichia canis*, *E. chaffeensis,* and *E. ewingii* (Dumler *et al*., 2001; Shaw *et al*., 2001). These Gram-negative obligate intracellular bacteria present a marked tropism for myeloid derivatives, multiplying within a membrane-bound cytoplasmic vacuole of the host cell and forming characteristic mulberry shaped microcolonies (morulae). Depending on the bacterial species, monocytes/macrophages (*E. canis*, *E. chaffeensis*), granulocytes (*A. phagocytophilum*, *E. ewingii*), or platelets (*A. platys*) are infected by these microorganisms. Acute Anaplasmataceae infections are multisystemic disorders generically characterized by a non-specific febrile illness with depression, lethargy, anorexia, splenomegaly,

hepatomegaly, lymphadenopathy and hematological abnormalities (Shaw *et al*., 2001). Persistent infection is a common feature of many of these organisms in canines, which in some cases may evolve into chronic disease. Canine monocytic ehrlichiosis, caused by *E. canis*, was the first canine Anaplasmataceae infection to be documented and is also one of the most important due to the number of deaths that occur with chronic disease (Donatien & Lestoquard, 1935; Huxsol, 1970; Milonasky *et al*., 2004; Gal *et al*., 2007).

In European dogs, only *A. phagocytophilum*, *A. platys* and *E. canis* have been isolated or detected by molecular tests (Johansson *et al*., 1995; Sparagano *et al*., 2003; Aguirre *et al*., 2004). In Portugal, serology has long provided evidence for *E. canis* infection in both stray and clinically ill dogs (Silveira, 1992; Bacellar *et al*., 1995). Additionally, *E. canis* DNA was recently identified in a case study of 55 dogs with suspected tick-borne disease, confirming canine monocytic ehrlichiosis in approximately one third of the cases (Alexandre, 2006). In the Alexandre study, a number of other tick-borne diseases such as babesiosis, hepatozoanosis, Lyme borreliosis and *Rickettsia conorii* infections were also identified in some cases (Alexandre, 2006). However, the majority of dogs remain undiagnosed. Knowing that *A. phagocytophilum* is present in Portuguese ticks (Santos *et al*., 2004) the objective of this work was to retest the available samples from those animals in order to investigate the possible role of *A. phagocytophilum* in canine disease.

## MATERIAL AND METHODS

The dogs included in this study presented with clinical signs between February and October 2004 among one hospital and seven veterinary clinics located in the Algarve region, in the South of Portugal. During the clinical evaluation, an EDTA-anticoagulated blood sample was collected from each animal for laboratory testing that included complete blood cell count, buffy coat smear examination after Diff-Quik staining (Medion Diagnostics GmbH, Germany), detection of specific antibodies by immunofluorescence assay (IFA), and DNA analysis by polymerase chain reactions (PCR). Whenever possible, a convalescent blood sample was taken to compare the evolution of IFA titers. The procedure adopted for laboratory diagnosis of *Babesia canis*, *Borrelia burgdorferi s.l.*, *Hepatozoon canis*, *E. canis*, and *Rickettsia* spp. is described elsewhere (Alexandre, 2006). For *A. phagocytophilum* testing, plasma samples were screened by IFA at an initial dilution of 1:80. Sera were incubated on antigen slides prepared with *A. phagocytophilum*

Webster strain, as described elsewhere (Santos *et al*., in submission). Fluorescein isothiocyanate (FITC)-conjugated rabbit anti-dog immunoglobulin G (IgG) and M (IgM) (Sigma-Aldrich, Germany) was used to identify bound antibodies that resulted in homogeneous fluorescence of *A. phagocytophilum* morulae. All positive samples were serial diluted to determine the endpoint titer. Serum from a naturally infected dog in the US and phosphate-buffered saline (PBS) were used as controls. Genomic DNA was extracted from the buffy coat following the Flexigene DNA Kit procedure (Qiagen GmbH, Germany). Molecular analysis was performed as nested or single tube PCR reactions using several sets of primers that target three *A. phagocytophilum* genes: GE3a/GE10r followed by GE9f/GE2 for the amplification of a 546-bp sequence of *rrs* (16S rRNA gene) (Massung *et al*., 1998), HS1/HD6 followed by HS43/HS45 for the amplification of a 442-bp sequence of heat-chock operon, *groESL* (Sumner *et al*., 1997), and the species-specific MSP465f/MSP980r for the amplification of a 550-bp hypervariable region of surface protein genes, *msp2* (Caspersen *et al*., 2002). PCR was performed in a total volume of 50 μl, containing 1 μM of each primer, 2.5 U of Taq DNA polymerase and 200 μM of each deoxynucleotide triphosphate and 50 mM KCl 10 mM Tris-HCL pH 8.3, and 1.5 mM $Mg^{2+}$ (Eppendorf MasterTaq Kit, Germany), and 10 μl DNA extract (or 1 μl of amplicon in nested reactions). Primer sequences and amplification conditions have been previously described (Sumner *et al*., 1997; Massung *et al*., 1998; Caspersen *et al*., 2002). DNA extracts obtained from the buffy coat of a healthy dog and *A. phagocytophilum* Webster strain propagated in HL-60 cells were used as controls. Since *rrs* and *groESL* primers are able to amplify DNA from other related bacteria besides *A. phagocytophilum*, PCR results from all positive reactions were confirmed by sequence analysis after DNA purification (Jetquick PCR Purification Kit; Genomed GmbH, Germany). Sequencing was performed with the forward and reverse primers used for PCR identification (internal primers in nested reactions) in an ABI automated sequencer (Applied Biosystems), according to manufacturer's instructions. After review and editing, sequence homology searches were made by BLASTN analysis. Sequences derived from this approach were aligned using the neighbor joining protocol with other Anaplasmataceae sequences for *rrs* and *groESL* using ClustalX. After trimming sequences to compare those of similar length, the sequences were re-aligned with ClustalX using the neighbor joining method, with 1000 replicate bootstraps. The resulting trees were produced using TreeView, rooting with *E. coli* as an outgroup for *rrs* and *R. rickettsii* as an outgroup for *groESL*. Partial gene sequences generated in this work are deposited in GenBank under the accession numbers EU004823 (*rrs*), EU004824 and EU004825 (*groESL*). Partial nucleotide sequences determined for *groEL* were

translated into amino acid sequences to determine whether the low level nucleotide heterogeneity identified had an impact on protein structure. To do this, the translated amino acid sequences were aligned (using ClustalX), and truncated so that each began with the initial methionine; dendrograms were constructed using TreeView.

## RESULTS

Of 55 dogs with suspected tick-borne disease, 30 (54.5%) had at least one plasma sample with detectable IgG antibodies against *A. phagocytophilum*. The endpoint titers were 80 for 12 samples, 320 for 11 samples, 640 for 4 samples, 1280 for 5 samples, 2560 for 2 samples, 5120 and 10240 for 1 sample each, altogether 36 positive samples. No IgM antibodies against *A. phagocytophilum* were detected in studied animals. In dogs with more than one available sample, 25% (5/20) fulfilled the serologic criteria for active infection, presenting seroconversion (No. 1, 3, 5 and 8) or four-fold increase in antibody titer (No. 6) (Table 1). PCR amplification with *rrs* and *groESL* primers generated products of the expected size in buffy coat samples from 5 dogs, including 3 seronegative animals (No. 4, 7 and 9), 1 which seroconverted (No. 5) and 1 that had a single positive IFA titer (No. 2). No sample was found PCR positive when tested with *A. phagocytophilum* species-specific *msp2* primers. Nucleotide analysis of *rrs* and *groESL* amplicons confirmed this latter result and identified the etiologic agent to be the closely related species *A. platys*. Partial sequences of *rrs* (GenBank accession no. EU004823), excluding the primers regions were identical in all dogs and were 100% (496/496 bp) homologous with previously reported *A. platys* sequences, such as GenBank accession number AY530806 that was identified in a Spanish dog (Figure 1A). Sequence polymorphisms were observed in *groEL* and resulted in 2 different genotypes. DNA nucleotide sequences 99.3% (438/441) and 100% (441/441) similar to *A. platys* described in Venezuela (AF399916) were detected in dogs 2 and 9 (GenBank accession no. EU004825) and in dogs 4, 5 and 7 (GenBank accession no. EU004824), respectively (Figure 1B). Thus, despite the minimal heterogeneity observed at the nucleotide level, the GroEL proteins produced by each of the *A. platys* infecting these Portuguese dogs were identical in the regions examined (Figure 2). Although the *A. platys* positive PCR results, no morulae were found within platelets on blood smear examination. Moreover, the results from other tick-borne agents molecular tests also revealed coinfections. Dog 7 that was previously identified actively infected

with *R. conorii* was also found infected with *A. platys*. Additionally, three dogs (No. 1, 6 and 8) with either *R. conorii* or *E. canis* DNA also fulfilled IFA criteria for active *A. phagocytophilum* infection, although all were *A. platys* PCR negative. Clinical examination revealed that all dogs with molecular confirmation of *A. platys* infection had lymphadenopathy accompanied in the majority of cases by fever (except in dog 9). The most frequent haematological abnormality was thrombocytopenia (5/5 dogs) and anaemia (4/5 dogs). Clinical and laboratory data of dogs fulfilling serodiagnostic criteria for active infection or positive PCR results are summarized in table I.

## DISCUSSION

*Anaplasma phagocytophilum* (formerly *Ehrlichia phagocytophila*, *E. equi* and HGE agent) is the etiologic agent of worldwide veterinary tick-borne disease that affects several domestic animals including ruminants, horses, dogs and cats, but it is also regarded as an emerging human pathogen with increasing importance (Gordon *et al*., 1932; Gribble, 1969; Madewell & Gribble, 1982; Bakken *et al*., 1994; Chen *et al*., 1994; Bjoerdorf *et al*., 1999). The disease is generically known as granulocytic anaplasmosis (GA) and in dogs it was first recognized by Madewell and Gribble (1982). The geographic distribution of GA in dogs correlates with the endemic areas of the vector ticks, members of the *Ixodes persulcatus/ricinus* complex. The disease is frequently reported in the Northeastern and Upper Midwest parts of United States and in Central and Northern Europe, but there are also some references to its presence in the Mediterranean Region (Manna *et al*., 2004; Alberti *et al*., 2005). The recent description of *A. phagocytophilum* in Portuguese *Ixodes* ticks (Santos *et al*., 2004) suggested a possible role for human and animal infection in Portugal, stimulating further examination of populations with suspected tick-borne disease. In this report we present the cytological, serological and molecular screening of *A. phagocytophilum* infections in a population of dogs with clinical signs who resided in the Algarve region, South of Portugal.

Serological data obtained by IFA testing demonstrated the presence of antibodies against *A. phagocytophilum* in 40% of animals studied, 25% of which fulfilled case criteria for active infection, either by seroconversion or four-fold increase in titer. Despite these findings, the molecular analysis of buffy coat samples failed to identify *A. phagocytophilum* but instead revealed sequences that were highly homologous with the closely related *A. platys*. Serologic cross-

reactivity is common among phylogenetically-related agents that often share antigenic determinants. In fact, at least some of the *A. phagocytophilum* IFA-positive sera detected here were derived from dogs with active *A. platys* infections, a finding also documented by others (Inokuma *et al*., 2001). Thus it seems likely that *A. platys* rather than *A. phagocytophilum* is the agent responsible for these serologic reactions, although prior infections can not be conclusively excluded. The possibility of cross-reactions with heterologous, Anaplasmataceae, such as *E. canis,* should also be considered. Cross-reactivity between the *E. canis* and *A. phagocytophilum* has been experimentally demonstrated in dogs with canine monocytic ehrlichiosis by the detection of antibodies against *A. phagocytophilum* about 90 days post-infection (Waner *et al*., 1998). It was proposed that the development of the cross-reacting antibodies was dependent on persistence of *E. canis* infection (Waner *et al*., 1998). In our study both dogs no. 1 and no. 8 seroconverted for *A. phagocytophilum* but had only *E. canis* DNA in the peripheral blood.

*A. platys* (formerly *Ehrlichia platys*) was first recognized in the US by Harvey and coworkers (1978) as a cause of canine infectious cyclic thrombocytopenia (CICT). This member of the family Anaplasmataceae is distributed worldwide, particularly associated with the brown dog tick *Rhipicephalus sanguineus*, one of the most prevalent species in Portugal (Caeiro, 1999). In fact, *A. platys* infections were previously suggested by the occasional observation of intra-platelet inclusions on dog peripheral blood smears (Simões & Puente, 1997), but its presence was never definitively proved. This study represents the first documentation of active *A. platys* infections in Portuguese dogs, although in our case no morulae were observed in blood smear examinations. The cyclical nature of CICT, with intermittent bacteremia may increase the difficulty in detection of the agent in peripheral blood (Woodly *et al*., 1991; Martin *et al.*, 2005). This could explain not only the inability to directly visualize the bacteria in PCR-confirmed cases, but could also explain negative molecular results in dogs with positive *A. phagocytophilum* serology.

CICT is usually described as an asymptomatic or mild disease without obvious clinical signs in spite of the occasional presence of lymphadenopathy and thrombocytopenia (Harvey *et al*., 1978; Woodly *et al*., 1991). In our case series, both these hematological findings were present in all confirmed *A. platys*-infected dogs, although the health condition of the animals was sufficiently deteriorated to motivated a visit to the veterinarian. A few other reports also linked CICT to clinical illness, either as a result of *A. platys* infection alone or as a coinfection with other tick-borne agents such as *E. canis* (Mylonakis *et al*., 2004; Gal *et al*., 2007). Although most of the present cases apparently represent single infections, DNA from both *A. platys* and *R. conorii* was,

detected in one animal. To our knowledge this study represents the first molecular evidence that *A. platys* infects and causes disease in Portuguese dogs. It further supports the hypothesis that coinfections with multiple tick-borne agents are not uncommon for those that share tick vectors such as *R. sanguineus*. The extent to which coinfections potentiate disease manifestations or complicate the diagnosis and management of sick dogs will require further study.

## ACKNOWLEDGEMENTS

This research was partially supported by the Portuguese government through the *Fundação para a Ciência e a Tecnologia* grant BD/8610/2002. We thank to to the staff of the *Unidade Laboratorial de Utilização Comum*, *Instituto Nacional de Saude Dr. Ricardo Jorge*, for their valuable assistance with sequencing. Also a special thanks to all veterinarians directly involved in sample collection.

## REFERENCES

1. AGUIRRE E, SAINZ A, DUNNER S, AMUSATEGUI I, LOPEZ L, RODRIGUEZ-FRANCO F, LUACES I, CORTES O, TESOURO MA. First isolation and molecular characterization of *Ehrlichia canis* in Spain. Vet Parasitol. 2004;125(3-4):365-72.

2. ALBERTI A, ZOBBA R, CHESSA B, ADDIS MF, SPARAGANO O, PINNA PARPAGLIA ML, CUBEDDU T, PINTORI G, PITTAU M. Equine and canine *Anaplasma phagocytophilum* strains isolated on the island of Sardinia (Italy) are phylogenetically related to pathogenic strains from the United States. Appl Environ Microbiol. 2005;71(10):6418-22.

3. ALEXANDRE N. [Estudo clínico e epidemiológico da febre botonosa, ehrlichiose canina e borreliose de Lyme numa população de canídeos domésticos do Algarve]. Tese de Mestrado em Saúde Pública Veterinária, Faculdade de Medicina Veterinária, Universidade Técnica de Lisboa, Lisboa, 2006. [MSc Thesis in Portuguese and abstract in English].

4. BACELLAR F, DAWSON JE, SILVEIRA CA, FILIPE AR. Antibodies against Rickettsiaceae in dogs of Setubal, Portugal. Cent Eur J Public Health. 1995;3(2):100-2.

5. BAKKEN JS, DUMLER JS, CHEN SM, ECKMAN MR, VAN ETTA LL, WALKER DH. Human granulocytic ehrlichiosis in the upper Midwest United States: a new species emerging? JAMA. 1994;272:212–8.

6. BJOERSDORFF A, SVENDENIUS L, OWENS JH, MASSUNG RF. Feline granulocytic ehrlichiosis- a report of a new clinical entity and characterisation of the infectious agent. J Small Anim Pract. 1999;40(1):20-4.

7. CAEIRO V. General review of tick species present in Portugal. Parassitologia. 1999;41(Suppl 1):11-5.

8. CASPERSEN K, PARK J-H, PATIL S, DUMLER JS. Genetic variability and stability of *Anaplasma phagocytophila msp2* (*p44*). Infect Immun. 2002;70(3):1230-4.

9. CHEN S, DUMLER JS, BAKKEN JS, WALKER DH. Identification of a granulocytic *Ehrlichia* species as the etiologic agent of human disease. J Clin Microbiol. 1994;32:589–95.

10. DUMLER JS, BARBET AF, BEKKER CPJ, DASCH GA, PALMER GH, RAY SC, RIKIHISA Y, RURANGIRWA FR. Reorganization of genera in the families Rickettsiaceae and Anaplasmataceae in the order Rickettsiales: unification of some species of *Ehrlichia* with *Anaplasma*, *Cowdria* with *Ehrlichia* and *Ehrlichia* with *Neorickettsia*, description of six new species combinations and designation of *Ehrlichia equi* and 'HGE agent' as subjective synonyms of *Ehrlichia phagocytophila*. Int J Syst Evol Microbiol. 2001;51:2145-65.

11. DONATIEN A, LESTOQUARD F. Existence en Algérie d'une *Rickettsia* du chien. Bull Soc Pathol Exot. 1935;28:418-9.

12. GAL A, LOEB E, YISASCHAR-MEKUZAS Y, BANETH G. Detection of *Ehrlichia canis* by PCR in different tissues obtained during necropsy from dogs surveyed for naturally occurring canine monocytic ehrlichiosis. Vet J. 2007. In press.

13. GORDON WS, BROWNLEE A, WILSON DR, MACLEOD J. Tick-borne fever hitherto undescribed disease of sheep. J Comp Path Ther. 1932;45:301-7.

14. GRIBBLE DH. Equine ehrlichiosis. J Am Vet Med Assoc. 1969;155(2):462-9.

15. HARVEY JW, SIMPSON CF, GASKIN JM. Cyclic thrombocytopenia induced by a *Rickettsia*-like agent in dogs. J Infect Dis. 1978;137(2):182-8.

16. HUXSOLL DL, HILDEBRANDT PK, NIMS RM, AMYX HL, FERGUSON JA. Epizootiology of tropical canine pancytopenia. J Wildl Dis. 1970;6(4):220-5.

17. INOKUMA H, OHNO K, ONISHI T, RAOULT D, BROUQUI P. Detection of ehrlichial infection by PCR in dogs from Yamaguchi and Okinawa Prefectures, Japan. J Vet Med Sci. 2001;63(7):815-7.

18. JOHANSSON KE, PETTERSSON B, UHLEN M, GUNNARSSON A, MALMQVIST M, OLSSON E. Identification of the causative agent of granulocytic ehrlichiosis in Swedish dogs and horses by direct solid phase sequencing of PCR products from the 16S rRNA gene. Res Vet Sci. 1995;58(2):109-12.

19. MADEWELL BR, GRIBBLE DH. Infection in two dogs with an agent resembling *Ehrlichia equi*. J Am Vet Med Assoc. 1982;180(5):512-4.

20. MANNA L, ALBERTI A, PAVONE LM, SCIBELLI A, STAIANO N, GRAVINO AE. First molecular characterization of a granulocytic *Ehrlichia* strain isolated from a dog in South Italy. Vet J. 2004;167(3):224-7.

21. MARTIN AR, BROWN GK, DUNSTAN RH, ROBERTS TK. *Anaplasma platys*: an improved PCR for its detection in dogs. Exp Parasitol. 2005;109(3):176-80.

22. MASSUNG RF, SLATER K, OWENS JH, NICHOLSON WL, MATHER TN, SOLBERG VB, OLSON JG. Nested PCR assay for detection of granulocytic ehrlichiae. J Clin Microbiol. 1998;36:1090–5.

23. MYLONAKIS ME, KOUTINAS AF, BREITSCHWERDT EB, HEGARTY BC, BILLINIS CD, LEONTIDES LS, KONTOS VS. Chronic Canine Ehrlichiosis (*Ehrlichia canis*): A Retrospective Study of 19 Natural Cases. J Am Anim Hosp Assoc. 2004;40(3):174-84.

24. SANTOS AS, AMARO F, SANTOS-SILVA M, SOUSA R, MATHIAS ML, RAMALHINHO MG, NUNCIO MS, ALVES MJ, BACELLAR F; DUMLER JS. Detection of antibodies against *Anaplasma phagocytophilum* in wild-rodents, Portugal. In submission.

25. SANTOS AS, SANTOS-SILVA MM, ALMEIDA VC, BACELLAR F, DUMLER JS. Detection of *Anaplasma phagocytophilum* DNA in *Ixodes* ticks (Acari: Ixodidae) from Madeira Island and Setubal District, mainland Portugal. Emerg Infect Dis. 2004;10(9):1643-8.

26. SPARAGANO OA, DE VOS AP, PAOLETTI B, CAMMA C, DE SANTIS P, OTRANTO D, GIANGASPERO A. Molecular detection of *Anaplasma platys* in dogs using polymerase chain reaction and reverse line blot hybridization. J Vet Diagn Invest. 2003; 15(6):527-34.

27. SHAW SE, DAY MJ, BIRTLES RJ, BREITSCHWERDT EB. Tick-borne infectious diseases of dogs. Trends Parasitol. 2001;17(2):74-80.

28. SILVEIRA CA. [Ehrlichiose canina. Estudo clínico de uma população animal, na região urbana e rural de setúbal – Implicações em Saúde Pública e Saúde Pública Veterinária]. Tese de Mestrado em Saúde Pública Veterinária, Faculdade de Medicina Veterinária, Universidade Técnica de Lisboa, Lisboa 1992. [MSc Thesis in Portuguese and abstract in English].

29. SIMÕES PB, PUENTE P. [Alguns hemoparasitas na rotina laboratorial: *Ehrlichia canis, E. platys, Hepatozoon canis* and *Babesia spp*]. Veterinária Técnica. 1997; 26-9. [Article in Portuguese].

30. SUMNER JW, NICHOLSON WL, MASSUNG RF. PCR amplification and comparison of nucleotide sequences from the *groESL* heat shock operon of *Ehrlichia* species. J Clin Microbiol. 1997;35:2087-92.

31. WANER T, STRENGER C, KEYSARY A, HARRUS S. Kinetics of serologic cross-reactions between *Ehrlichia canis* and the *Ehrlichia phagocytophila* genogroups in experimental *E. canis* infection in dogs. Vet Immunol Immunopathol. 1998;66(3-4):237-43.

32. WOODY BJ, HOSKINS JD. Ehrlichial diseases of dogs. Vet Clin N Am Small Anim Pract. 1991;21:75–98.

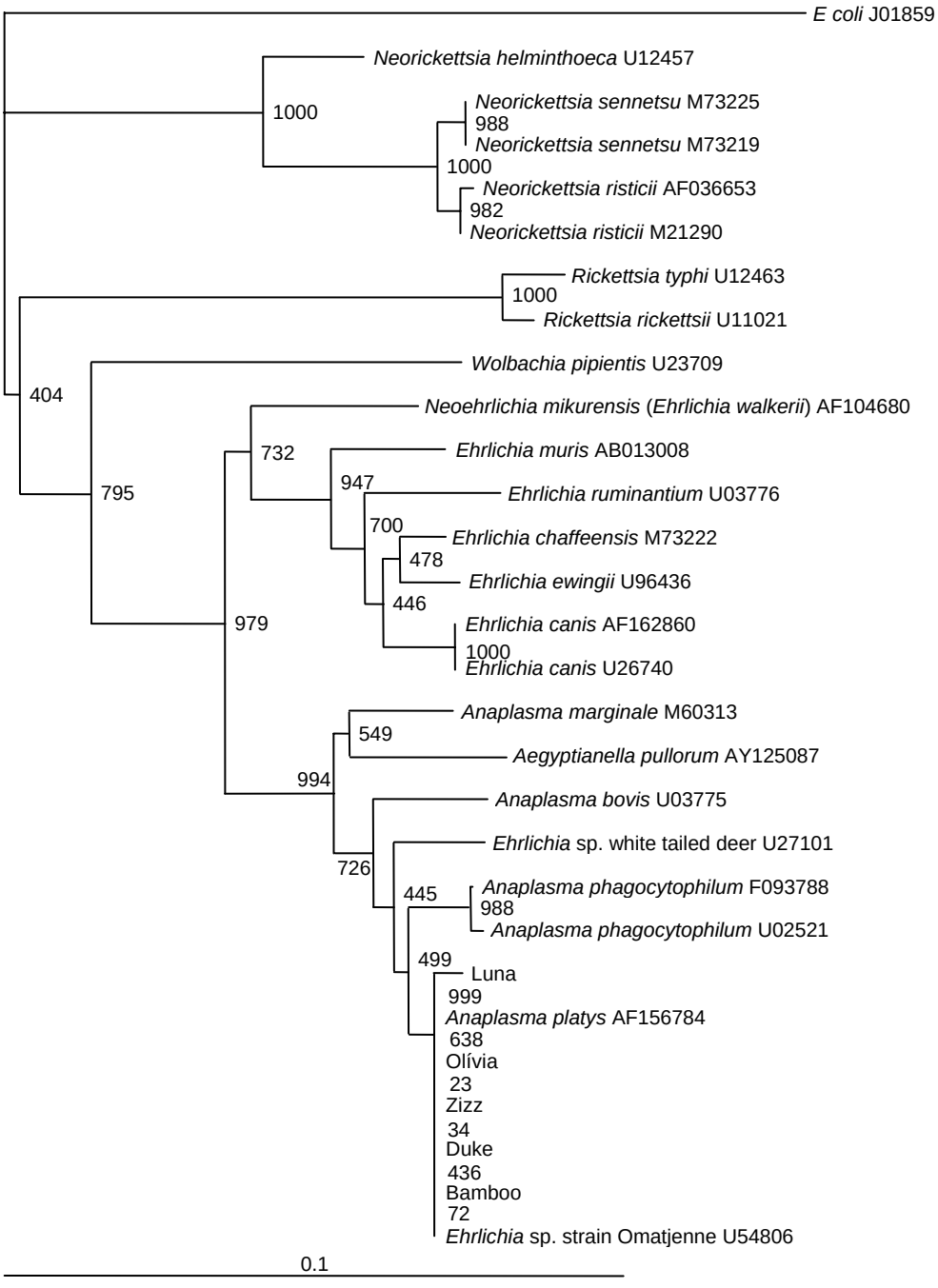
TABLE 1. Dogs presented with *Anaplasma* active infections detected by IFA and PCR.

| Dog No. | Sex[a] | Age[b] | Breed | Clinical signs | Hematologic abnormalities[c] | IFA[d] | *PCR*[f] | | | Other tick-borne infections[g] |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | *16S rDNA* | *groESL* | *Msp2* | |
| 1- Babylon | F | 8y | Cross-breed | Fever (39.4ºC), lymphadenopathy, pale mucosa | A, Lc, T | <80; 1,280 | - | - | - | Ec |
| 2- Bamboo | M | 3y | Cross-breed | Lymphadenopathy, pale mucosa, weight loss, diarrhea, vomit | A, T | 320 | + | + | - | - |
| 3- Black | F | 2 y | Pequinois | Fever (39.3ºC), lymphadenopathy, pale mucosa | A, T | <80; 320 | - | - | - | - |
| 4- Duke | M | 4y | Doberman | Fever (40.5ºC), lymphadenopathy, pale mucosa | A, T | <80 | + | + | - | - |
| 5- Luna | F | 1 y | Boxer | Fever (40.1 ºC), lymphadenopathy | T | <80; 80 | + | + | - | - |
| 6- Nina | F | 5y | Pincher | Fever (40.4), lymphadenopathy, pale mucosa | A, T | 80; 1280 | - | - | - | Rc |
| 7- Olívia | F | 3 m | Serra da Estrela Mountain Dog | Fever (39.6ºC), lymphadenopathy, pale mucosa | A, T | <80 | + | + | - | Rc |
| 8- Sniper | M | 1 y | Pittbull | Fever (41.4ºC), lymphadenopathy | A, L, T | <80; 80 | - | - | - | Ec |
| 9- Zizzi | M | 1y | Cross-breed | Fever (40.1ºC), lymphadenopathy, anorexia | A, Lc, T | <80 | + | + | - | - |

[a]M male, F female; [b]y year, m month; [c]A anemia *<5,5 x10$^6$ /µl*; Lc leukocytosis > 17 *x10$^3$ /µl*; L leukopenia <6 *x10$^3$ /µl*; T thrombocytopenia *<200 x10$^3$ /µl*; [d]IFA titer(s) of available plasma samples; [f]Genes tested by PCR; [g]Results of previous PCR testing has detected active infection with Ec *Ehrlichia canis* and Rc *Rickettsia conorii* (Alexandre, 2006).

FIGURE 1. Phylogenetic relationships between *A. platys* sequences detected in this study other Anaplasmataceae based on partial nucleotide sequences of the *rrs* (A) and *groESL* (B). The scale bar represents substitution rates of 10 per each 100 nucleotides.

A) *rrs*



B) *groESL*

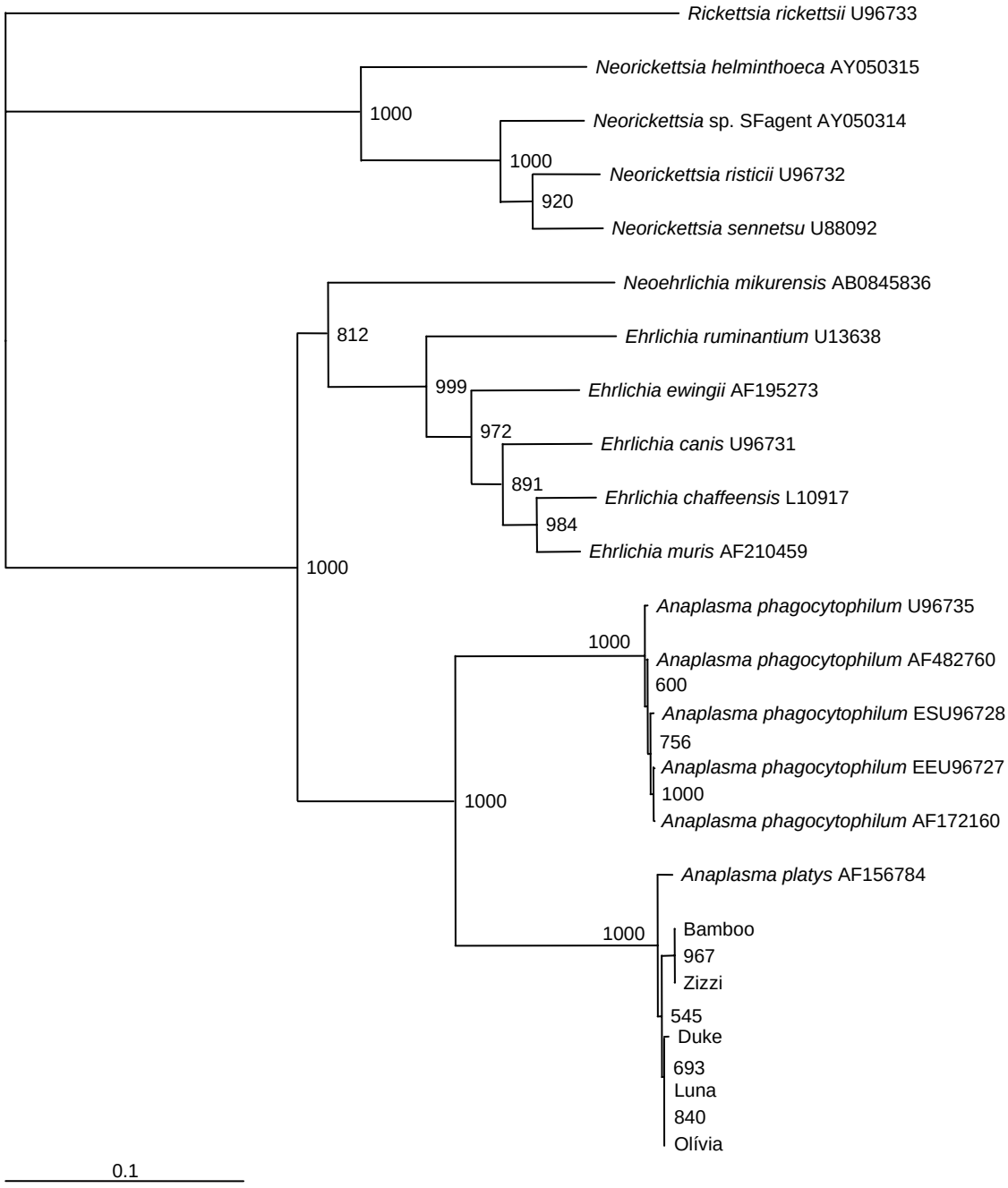
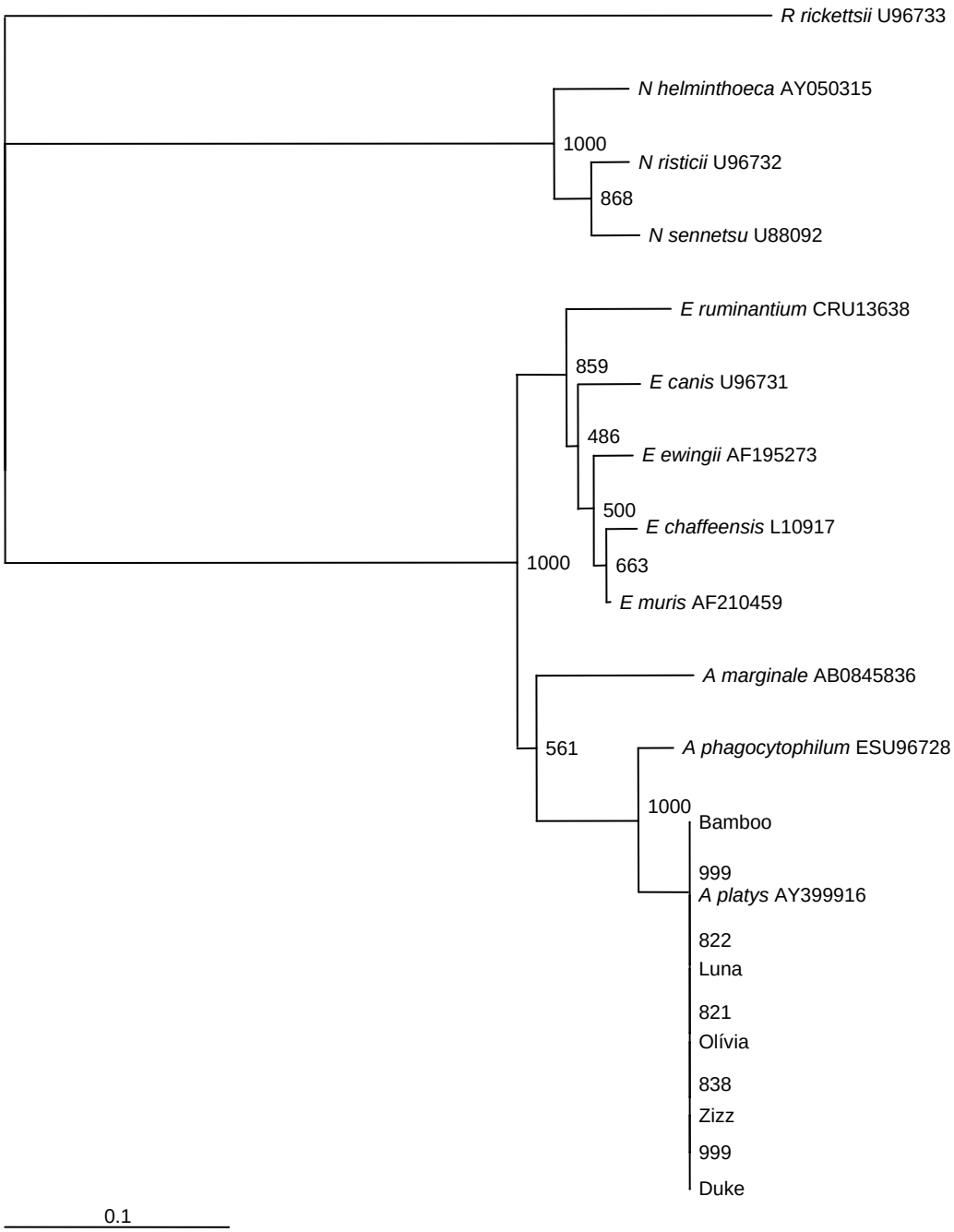

*Rickettsia rickettsii* U96733
*Neorickettsia helminthoeca* AY050315
1000
*Neorickettsia* sp. SFagent AY050314
1000
*Neorickettsia risticii* U96732
920
*Neorickettsia sennetsu* U88092
*Neoehrlichia mikurensis* AB0845836
812
*Ehrlichia ruminantium* U13638
999
*Ehrlichia ewingii* AF195273
972
*Ehrlichia canis* U96731
891
*Ehrlichia chaffeensis* L10917
984
*Ehrlichia muris* AF210459
1000
*Anaplasma phagocytophilum* U96735
1000
*Anaplasma phagocytophilum* AF482760
600
*Anaplasma phagocytophilum* ESU96728
756
*Anaplasma phagocytophilum* EEU96727
1000
*Anaplasma phagocytophilum* AF172160
1000
*Anaplasma platys* AF156784
1000
Bamboo
967
Zizzi
545
Duke
693
Luna
840
Olívia
0.1

FIGURE 2. Dendrogram demonstrating the similarity among GroEL amino acid sequences derived from *A. platys*-infected Portuguese dogs compared to other Anaplasmataceae species. Note that despite the nucleotide heterogeneity found among the Portuguese dogs (Figure 1), the amino acid sequences and therefore GroEL protein structure are predicted be identical.



# CHAPTER V

## HUMAN EXPOSURE TO *ANAPLASMA PHAGOCYTOPHILUM*

# 5.1. HUMAN EXPOSURE TO *ANAPLASMA PHAGOCYTOPHILUM* IN PORTUGAL

SANTOS AS, BACELLAR F, DUMLER JS

SHORT REPORT

ANNALS OF NEW YORK ACADEMY OF SCIENCES 2006; 1078:100-05

# Human Exposure to *Anaplasma phagocytophilum* in Portugal

A.S. SANTOS,[a] F. BACELLAR,[a] AND J.S. DUMLER[b]

[a]*Centro de Estudos de Vectores e Doenças Infecciosas (CEVDI), Instituto Nacional de Saúde Dr. Ricardo Jorge, Águas de Moura, Portugal*

[b]*Division of Medical Microbiology, Department of Pathology, The Johns Hopkins University School of Medicine, Baltimore, Maryland, USA*

**ABSTRACT: A retrospective study to detect *Anaplasma phagocytophilum* antibodies by indirect immunofluorescence (IFA) and Western blot (WB) assay was conducted in 367 potentially exposed patients from Portugal. The study included 26 patients with confirmed Lyme borreliosis (LB), 77 with suspected LB, 264 seronegative patients studied for possible tick-transmitted LB and boutonneuse fever (LB/BF) infection, and 96 healthy blood donors. Overall, patients with LB and suspected LB ($n = 2$ [7.7%] and $n = 6$ [7.8%], respectively) were more often seropositive ($n = 8$ [7.8%]; $P < 0.001$), whereas only 1 (0.4%; $P = 0.046$) patient in the LB/BF seronegative group had confirmed disease. This study is the first evidence of human exposure to *A. phagocytophilum* or an antigenically similar bacterium in Portugal, and suggests that LB patients are significantly more likely to contact *A. phagocytophilum*.**

**KEYWORDS: serologic study; *Anaplasma phagocytophilum* exposure; Lyme borreliosis; Portugal**

## INTRODUCTION

Human granulocytic anaplasmosis (HGA) is an emerging tick-borne illness caused by *Anaplasma phagocytophilum*. In Portugal, the bacterium is present in both *Ixodes ricinus* and *I. ventalloi* ticks,[1] but no data concerning the occurrence of HGA are available. Because *I. ricinus* frequently bite humans, we conducted a retrospective study of *A. phagocytophilum* antibodies in potentially exposed patients.

Address for correspondence: Ana Sofia Santos, Centro de Estudos de Vectores e Doenças Infecciosas, Instituto Nacional de Saúde Dr. Ricardo Jorge, Avenida da Liberdade 5, 2965-575 Águas de Moura, Portugal. Voice: +351-265-912222; fax: +351-265-912568; +351-265-912155.
e-mail: ana.santos@insa.min-saude.pt

**Ann. N.Y. Acad. Sci. 1078: 100–105 (2006).** 
**doi: 10.1196/annals.1374.014**

## MATERIAL AND METHODS

Sera from clinically ill individuals submitted to CEVDI's laboratory during 2002 for Lyme borreliosis (LB) and boutonneuse fever (BF) testing were used. The study included 26 patients with confirmed LB, 77 with suspected LB, 264 seronegative patients studied for possible tick-transmitted (LB/BF) infection, and 96 healthy blood donors. The first two cohorts included all confirmed and suspected LB cases with available sera.[2] The third cohort was a random sample comprising 25% of LB/BF seronegative patients detected monthly during the year. Institutional Review Board approval was obtained for these studies. Antibodies against *A. phagocytophilum* were detected by indirect immunofluorescence (IFA) and Western blot (WB) assays, as described previously.[3,4] *A. phagocytophilum* Webster and *Ehrlichia chaffeensis* Arkansas strains were used as antigen. *E. chaffeensis* cross-reactivity was evaluated in seropositive samples. The criteria for seropositivity were IFA titer $\geq$80 and WB reactivity with bands between 42–49 kDa for *A. phagocytophilum* and between 23–30 kDa for *E. chaffeensis*.[3,4] Confirmed *A. phagocytophilum* exposure was defined as a fourfold higher IFA titer to specific antigen than to *E. chaffeensis* or when IFA and WB were positive. Possible exposure was defined as a single IFA titer of $\geq$80 or less than fourfold titer difference between specific antigen and *E. chaffeensis*, and no specific WB bands. For statistical analysis, independence of proportions of reactive patient samples was compared between groups and the test population using the Chi-square test. $P$ values of $<0.05$ were considered significant.

## RESULTS

A total of 419 sera were obtained from 367 patients for serologic testing, not including 96 sera obtained from healthy blood donors. In 323 patients (88%) only one serum sample was available. Serologic results are summarized in TABLES 1 and 2 and in FIGURE 1. Confirmed and possible exposures to *A. phagocytophilum* were present in 1 (1%) and 3 (3%) of 96 blood donors, respectively. Patients with possible exposure were not more likely to have clinical findings ($P = 0.10$, $\chi^2$ test); thus, only confirmed patients were considered infected. Overall, 2.2% (10/463) individuals had confirmed exposure. Patients with LB or suspected LB were significantly more likely to have *A. phagocytophilum* confirmed ($P \leq 0.002$). Of 367 studied, patients with LB ($n = 2$ [7.7%]) and suspected LB ($n = 6$ [7.8%]) together ($n = 8$, [7.8%]) were more often seropositive ($P < 0.001$), whereas only one (0.4%; $P = 0.046$) patient in the LB/BF seronegative group had confirmation. *E. chaffeensis* cross-reactions were detected only in three patients and only in the suspected LB group. No statistically significant differences in age, gender, and province were noted between the *A. phagocytophilum*-confirmed and -seronegative individuals; the

**TABLE 1. Serologic testing results for *A. phagocytophilum* and *E. chaffeensis***

| Study groups[a] | No. tested patients/ no. reactive | No. confirmed (%) / no. possible (%) exposure | |
|---|---|---|---|
| | | *A. phagocytophilum* | *E. chaffeensis* |
| Patients with defined LB | 26/5 | 2(7.7) / 3(11.5) | — |
| Patients with suspected LB | 77/11 | 6 (7.8) / 2 (2.6) | — / 3(3.9) |
| Seronegative LB/BF patients | 264/4 | 1(0.4) / 3(1.1) | — |
| Blood donors | 96/4 | 1(1) / 3(3.1) | — |

[a] LB = Lyme borreliosis; BF = Boutonneuse fever.

median age of confirmed patients was 42 years, 66.6% were women and 55.5% came from Estremadura province. However, among patients in Estremadura province, *A. phagocytophilum*-confirmed cases more often resided in Oeiras than in other locations ($P < 0.001$).

## DISCUSSION

*A. phagocytophilum* is transmitted by *Ixodes* ticks and shares these vectors with other human infectious agents, such as *Borrelia burgdorferi* (etiologic agent of LB). This ecological characteristic of *A. phgocytophilum* allowed us to focus the study on patients with clinical and laboratory evidence of confirmed or suspected LB in order to detect potential HGA. HGA results in an acute, generally self-limited, nonspecific febrile illness, characterized by headache and myalgias, often accompanied by systemic symptoms and signs and hematological abnormalities such as thrombocytopenia, leukopenia, elevated serum hepatic transaminases, and/or elevated serum C-reactive protein concentrations. This nonspecific presentation is common with other tick-borne illnesses, justifying the inclusion of an additional patient group with clinical suspicion of LB or BF but lacking laboratory confirmation for these diagnoses. In this report we have found that significantly higher proportions of patients with suspected/confirmed LB had confirmed exposure to *A. phagocytophilum* than did the overall population. Our results corroborate previous findings, and show that the prevalence of *A. phagocytophilum* infection among Portuguese LB patients (7.8%) is in the range of 2–21% as described in other European countries.[5] However, the evaluation is partly impaired by the lack of analysis of paired sera that underestimates prevalence. Cultivation of Portuguese strains could also help to improve serologic tests. In conclusion, these data provide evidence that Portuguese *A. phagocytophilum* strains infect and evoke immune responses in tick-exposed humans, and that LB patients are significantly more likely to acquire *A. phagocytophilum* infection. The possibility of coinfection with both pathogens and comorbidity is worthy of clinical consideration.

**TABLE 2. Epidemiological and clinical data from patients with confirmed exposure to *A. phagocytophilum***

| Patient groups[a] | | Gender[b]/ age (year)[c] | Origin of residence[d] | Clinical presentation/ laboratory findings[e] | Onset (days) | No. sera tested (time interval)[f] | *A. phagocytophilum* serology | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | IFA titer (Ig class) | WB result[g] |
| Defined LB | 1 | F/63 | Elvas; Alto Alentejo | Febrile illness/— | — | 2 (68 days) | 80(IgG); 1280(IgG) | − |
| | 2 | F/57 | Oeiras; Estremadura | — | — | 1 | 640(IgG) | + |
| Suspected LB | 3 | M/U | Algarve | — | — | 2 (13 days) | 320(IgG) | + |
| | 4 | M/74 | Oeiras; Estremadura | — | — | 1 | 320(IgG) | + |
| | 5 | F/67 | Lisboa; Estremadura | Vasculitis | — | 1 | 320(IgM) | + |
| | 6 | F/9 | Oeiras Estremadura | — | — | 1 | 160(IgG) | + |
| | 7 | M/50 | Oeiras; Estremadura | — | — | 1 | 160(IgM) | + |
| | 8 | F/31 | V F Xira; Ribatejo | — | — | 3 (112 days) | 80(IgG) | + |
| Seronegative LB/BF | 9 | F/19 | F Foz; Beira Litoral | Febrile illness/ LT | 8 | 1 | 80(IgM) | + |

[a]LB = lyme borreliosis; BF = boutonneuse fever; [b]M = male; F = female; [c]U = unknown; [d]Patient locality and province; [e]LT = Elevated liver transaminases; [f]Average in days between consecutive sera samples; [g]Western blot result for polyvalent antibodies testing.

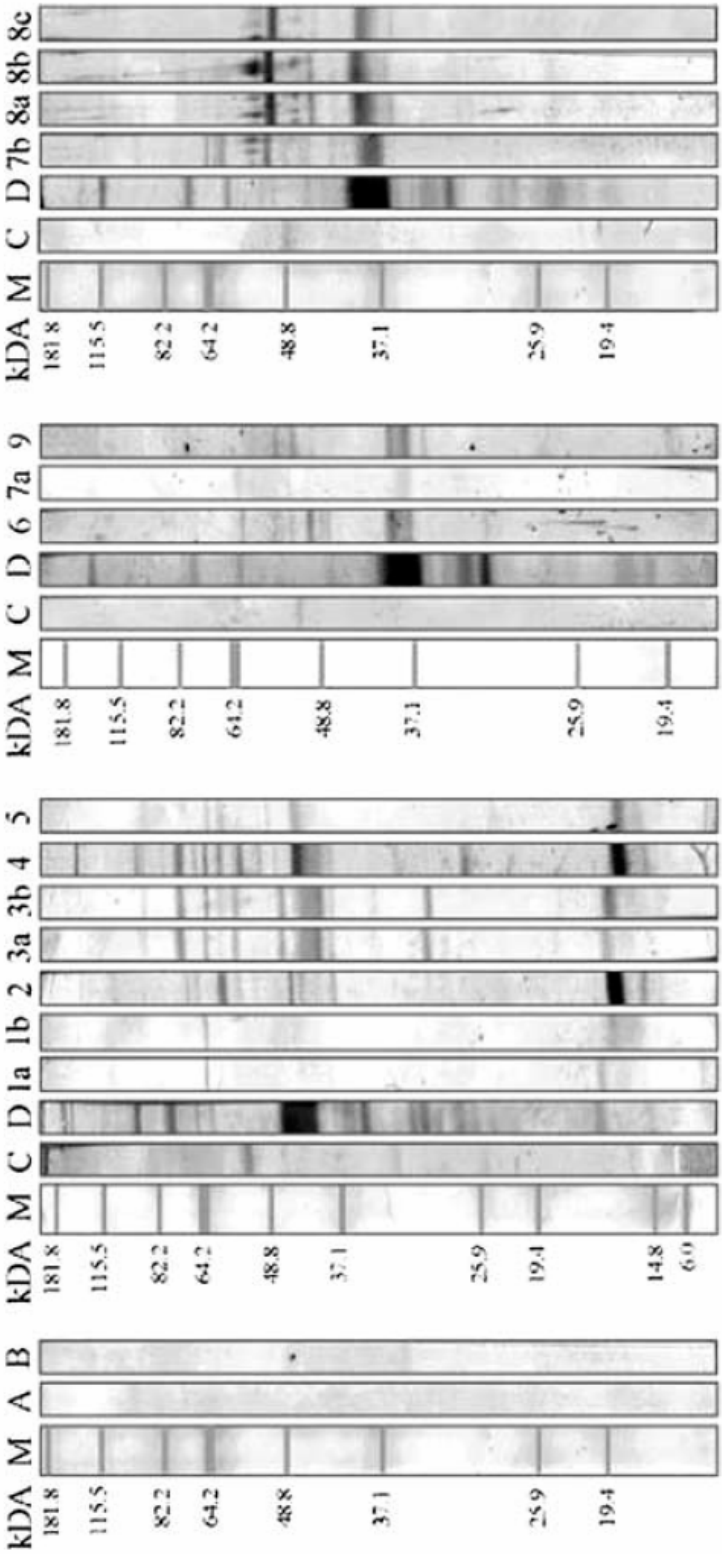



**FIGURE 1.** Western blot results for *A. phagocytophilum* IFA-positive patients.

## ACKNOWLEDGMENTS

This research was partially supported by the Portuguese government through Fundação para a Ciência e a Tecnologia Grant BD/8610/2002 and through the National Institutes of Health Grant R01-AI41213 to JSD. A special thanks to Cevdi and Dumler's lab team.

## REFERENCES

1. Santos, A.S. *et al*. 2004. Detection of *Anaplasma phagocytophilum* DNA in *Ixodes* ticks (Acari: Ixodidae) from Madeira Island and Setúbal District, Mainland Portugal. Emerg. Infect. Dis. **10:** 1643–1648.
2. Lopes de Carvalho, I., T. Luz & M.S. Núncio. 2004. 14 year follow-up study of Lyme borreliosis in Portugal. Presented at the third Congress of the European Society for Emerging Infections. Paris, France. October 17–20.
3 Wong, S.J., G.S. Brady & J.S. Dumler. 1997. Serologic responses to *Ehrlichia equi*, *Ehrlichia chaffeensis* and *Borrelia burgdorferi* in patients from New York State. J. Clin. Microbiol. **35:** 2198–2205.
4. Unver, A. *et al*. 2001. Western blot analysis of sera reactive to human monocytic ehrlichiosis and human granulocyic ehrlichiosis agents. J. Clin. Microbiol. **39:** 3982–3986.
5. Blanco, J.R. & J.A. Oteo. 2002. Human granulocytic ehrlichiosis in Europe. Clin. Microbiol. Infect. **8:** 763–772.

## 5.2. SIX YEARS OF ANAPLASMATACEAE SERODIAGNOSIS IN HUMANS. DATA FROM A STATE LABORATORY, PORTUGAL

SANTOS AS, DE SOUSA R, LOPES DE CARVALHO I, NÚNCIO MS, DUMLER JS, BACELLAR F

RESEARCH ARTICLE

IN SUBMISSION

.

# Six years of Anaplasmataceae serodiagnosis in humans. Data from a state laboratory, Portugal

Santos AS[1], De Sousa R[1], Lopes de Carvalho I[1], Núncio MS[1], Dumler JS[2], Bacellar F[1]

[1]Centro de Estudos de Vectores e Doenças Infecciosas, Instituto Nacional de Saúde Dr. Ricardo Jorge, Lisboa, Portugal;
[2]Division of Medical Microbiology, Department of Pathology, The Johns Hopkins University School of Medicine, Baltimore, Maryland, USA.

Corresponding author: Ana Sofia Pereira dos Santos. Centro de Estudos de Vectores e Doenças Infecciosas, Instituto Nacional de Saúde Dr. Ricardo Jorge, Edifício LEMES, Avenida Padre Cruz, 1649-016 Lisboa, Portugal;
Tel: +351 217 508 125; Fax: +351 217 508 521
e-mail: ana.santos@insa.min-saude.pt

ABSTRACT

The present work summarizes the available data concerning Anaplasmataceae diagnosis, including *Anaplasma phagocytophilum* and *Ehrlichia chaffeensis*, performed at the the Center for Vector and Infectious Diseases Research, National Institute of Health Dr. Ricardo Jorge (*CEVI/INSA*) during 2000-2006. As part of routine diagnosis, *CEVDI/INSA* prospectively investigated the presence of antibodies by indirect immunofluorescent assay (IFA) against *E. chaffeensis* from 2000-2006 and against *A. phagocytophilum* in 2006. Sera archived in 2000-2005 from patients with suspected Anaplasmataceae infection were also retrospectively studied by IFA for *A. phagocytophilum*. Positive sera were subsequently screened by PCR to detect Anaplasmataceae active infections. Investigation of active infection by PCR and *in vitro* isolation were also performed on whole-blood samples submitted in 2006. A total of 619 sera from 425 patients was tested. Overall, antibodies to *E. chaffeensis* and *A. phagocytophilum* were detected in 5.6% (24/425) of patients. Serologic reactions to *A. phagocytophilum* were detected in 70.8% (17/24), to *E. chaffeensis* in 12.5% (3/24), or to both antigens in 16.6% (4/24) of cases. The majority had stable or less than four-fold changes in antibody titer between consecutive samples. *A. phagocytophilum* seroconversion was observed in only one patient, fulfilling criteria for recent infection, although this patient also seroconverted to *Coxiella burnetii*. Neither *A. phagocytophilum* nor *E. chaffeensis* active infections were detected by PCR. Laboratory testing for other tick-borne agents or related bacteria also provided evidence for bartonellosis and acute Q fever in one and five *A. phagocytophilum*-seropositive patients, respectively, for rickettsiosis in two *E. chaffeensis*-seropositive patients, and for acute and chronic Q fever in two patients with both *A. phagocytophilum* and *E. chaffeensis* antibodies. Moreover one patient with stable *A. phagocytophilum* titer seroconverted to *C. burnetii*, which was later isolated from the patient's blood sample. These findings could represent false positive cross-reactions to shared antigens, antibodies induced by active dual infections, or past exposures to Anaplasmataceae species and other agents. Regardless, the results argue for continued development of improved diagnostics and integrated analysis of diagnostic tests for patients with suspected tick-borne disease.

RUNNING TITLE

Human Anaplasmataceae serodiagnosis, Portugal

KEYWORDS

Laboratory diagnosis; Human Anaplasmataceae infections; *Ehrlichia chaffeensis*; *Anaplasma phagocytophilum*;

INTRODUCTION

Human infections caused by bacteria belonging to the family Anaplasmataceae (Class α-proteobacteria, Order Rickettsiales), represent some of the best examples of emerging vector-borne diseases. Sennetsu fever (SF), documented in Japan in 1954 (Misao & Kobayashi, 1955), was the first human disease known to be caused by a member of the Anaplasmataceae family. This usually mild mononucleosis-like illness is caused by *Neorickettsia* (formerly *Ehrlichia*) *sennetsu*, likely acquired after ingestion of raw fish infested by infected trematodes. The infection occurs only in limited areas of the Far East, rarely outside Japan and Malaysia, and no deaths have been reported.

In 1986, human monocytic ehrlichiosis (HME) was reported in a patient from Central Arkansas. The infection was caused by a previously unknown tick-borne agent, closely related to the animal pathogen *E. canis* that was later named *Ehrlichia chaffeensis* (Maeda *et al*., 1987; Anderson *et al.*, 1991; Dawson *et al*., 1991). Additionally, other tick-borne Anaplasmataceae of veterinary importance were also described as causes of human infection. In the early 1990's, cases of human granulocytic anaplasmosis (HGA) (formerly human granulocytic ehrlichiosis [HGE]) were reported from Minnesota and Wisconsin (Bakken *et al*., 1994; Chen *et al*., 1994). The exact nature of the causative agent, originally named "HGE agent", remained uncertain for several years until its unification with the animal pathogens *E. phagocytophila* and *E. equi* into a single species, *Anaplasma phagocytophilum* (Dumler *et al*., 2001, 2005).

,

In 1996, *E. canis* - the etiologic agent of canine monocytic ehrlichiosis was isolated from an apparently healthy man (Perez *et al*., 1996), and was later associated with several symptomatic infections (Perez *et al*., 2006) in Venezuela. More recently, in 1999 *E. ewingii*, the etiologic agent of canine granulocytic ehrlichiosis was also identified as a human disease agent in US, mostly in immunocompromised patients (Buller *et al*., 1999). Of the above human diseases caused by pathogens assigned to Anaplasmataceae family, HME and HGA are now considered the most important due to the number of cases annually reported in US, the potential for fatal outcome and the broad worldwide geographic distribution of the etiologic agents, especially in Europe.

In Portugal, the potential involvement of Anaplasmataceae species in human disease was first suggested by David de Morais and coworkers (1991), with the description of a case fulfilling serologic criteria for active *E. chaffeensis* infection. Additionally, a study performed on Madeira Island designed to clarify the importance of *Ixodes ricinus* as a vector of tick-borne agents showed for the first time *A. phagocytophilum* DNA in Portuguese *I. ricinus* ticks (Nuncio *et al*., 2000). A project was then initiated to evaluate whether *A. phagocytophilum* could be an important pathogen in the country and confirmed its presence not only on Madeira Island but also in mainland *Ixodes* ticks (Santos *et al*., 2004), and the presence of antibodies against this agent in patients with tick-borne diseases (Santos *et al*., 2006). The potential for human exposure to Anaplasmataceae in Portugal justified the implementation of laboratory diagnostics for these tick-borne agents by the Center for Vector and Infectious Diseases Research from the National Institute of Health Dr. Ricardo Jorge (*CEVDI/INSA*). This work summarizes all available data concerning *E. chaffeensis* and *A. phagocytophilum* diagnostics performed in *CEVDI/INSA* during 2000-2006.

## MATERIAL AND METHODS

Patient Samples. This study includes patients from which at least one biological sample, including whole-blood and/or serum, was received at *CEVDI/INSA* during 2000 to 2006 for laboratory diagnosis of *A. phagocytophilum* and/or *E. chaffeensis* infection. The samples were received either from state hospitals, public health centers, or directly from physicians practicing in diverse regions of the country.

Serology. An indirect immunofluorescent assay (IFA) was used for serologic diagnosis of

Anaplasmataceae infections. The presence of antibodies against *E. chaffeensis* was studied prospectively in all available sera as part of *CEVDI/INSA* routine diagnosis conducted from 2000-2005. For *A. phagocytophilum*, laboratory diagnosis was available only in 2006; thus, sera archived during 2000-2005 from patients with suspected Anaplasmataceae infection were studied retrospectively. Institutional Review Board approval was obtained for this retrospective study. In 2006, sera received for either *A. phagocytophilum* or *E. chaffeensis* testing were examined prospectively for antibodies against both agents. IFA was performed according to *CEVDI/INSA* procedures, as previously described (Santos *et al*., in submission). Briefly, sera were diluted in PBS and incubated for 30 min at 37ºC using commercial *A. phagocytophilum* and *E. chaffeensis* antigen slides (Focus Diagnostics, Cypress CA, USA). The retrospective study was performed using *A. phagocytophilum* Webster strain antigen slides prepared in-house (Santos *et al*., in submission). A second incubation for 30 min at 37ºC with fluorescein isothiocyanate (FITC)-labelled rabbit anti-human immunoglobulin M (IgM) and polyvalent IgA,G,M conjugates (Dako Cytomation, Denmark) were used to identify bound antibodies resulting in homogeneous fluorescence of Anaplasmataceae morulae. The IFA was interpreted as positive whenever titers of IgM ≥80 or IgA,G,M ≥80 for *A. phagocytophilum* or when IgM ≥32 or IgA,G,M ≥64 for *E. chaffeensis* were found. All reactive samples were serially diluted to determine the end-point titer, which was expressed as the reciprocal of the serum dilution. Positive and negative sera were included on every antigen slide as IFA controls.

Molecular analysis. Polymerase chain reaction (PCR) was used to attempt detection of *A. phagocytophilum* and *E. chaffeensis* DNA in EDTA-blood samples received at *CEVDI/INSA* during 2006. Additionally, Anaplasmataceae IFA-positive sera were tested by PCR. Since treatment information was missing on the majority of submission forms accompanying samples, molecular analysis was performed only on the first sample that more likely represented the acute or active phase of infection at time of presentation to the physician and prior to antibiotic therapy. Genomic DNA was extracted from 200 µl of buffy-coat or serum using the Qiamp Blood Kit procedure (Qiagen Gmbh, Germany). Known negative samples were included as controls with every set of 1 - 4 sample extractions. Nested or single tube PCR reactions targeting both the *A. phagocytophilum* and *E. chaffeensis groESL* operon, and *A. phagocytophilum rrs* (16S rRNA gene) and *msp2* (*p44*) genes were performed as previously described (Sumner *et al*., 1997; Massung *et al*., 1998;

Caspersen *et al*., 2002) in a total volume of 50 μl containing 1 μM of each primer, 2.5 U of Taq DNA polymerase, 200 μM of each deoxynucleotide triphosphate, 50 mM KCl 10 mM Tris-HCL pH 8.3, 1.5 mM $Mg^{2+}$ (Eppendorf MasterTaq Kit, Germany), and 10 μl DNA extract (or 1 μl of first stage amplicon in nested PCR reactions). In each run, known negative (including the extraction controls and water) and positive samples were used as controls. Prevention of cross-contamination was managed by using pipette tips with filter barriers, and by performing PCR in 3 separate rooms (extraction, master mix preparation, and amplification/ electrophoresis).

Isolation attempts. During 2006, isolation of *A. phagocytophilum* and *E. chaffeensis* was attempted from patients for whom an EDTA or heparinized blood sample was available at the time of their initial presentation, using the culturing methods previously described (Dawson *et al*., 1991; Goodman *et al*., 1996). Briefly, for *A. phagocytophilum* isolation, approximately 0.5 ml of whole-blood or 0.2 ml of crude buffy coat recovered after blood sedimentation by gravity or low speed centrifugation, was directly inoculated into flasks with HL-60 cells maintained at a final concentration of $2x10^5$ cells/ml in RPMI 1640 medium (Gibco, Invitrogen™, UK), supplemented with 1% FBS (Gibco, Invitrogen™, UK) and 2 mM L-glutamine (Gibco, Invitrogen™, UK). The cultures were incubated at 37ºC in a 5% $CO_2$ atmosphere and checked twice weekly to adjust cell concentrations and to investigate infection. Microbial growth was ascertained by microscopy of cytocentrifuged culture aliquots stained by Diff-Quik (Medion Diagnostics GmbH, Germany). *E. chaffeensis* isolation was performed by direct inoculation of whole-blood or crude buffy coat onto monolayers of DH82 cells, maintained in minimum essential medium (MEM; Gibco, Invitrogen™, UK) supplemented with 10% FBS, 2 mM L-glutamine (Gibco, Invitrogen™, UK), 2 mM sodium pyruvate (Gibco, Invitrogen™, UK) and non-essential aminoacids (MEM NEAA; Gibco, Invitrogen™, UK) at 37ºC and 5% $CO_2$. Twice weekly, the medium was changed and infection monitored as described above.

Differential Diagnosis. For Anaplasmataceae IFA-positive patients, when requested by the physician, data concerning *CEVDI/INSA* tested for other tick-borne agents or related bacteria such as *Bartonella* spp., *Borrelia burgdorferi sensu lato*, *Rickettsia* spp., and *Coxiella burnetii* were also included in the presented work. Routine laboratory diagnosis of active infections by the latter agents depended on the type of sample submitted, but was usually based on serology (IFA, ELISA;

or WB), molecular analyses (PCR) and/or isolation attempts. Based on the agent suspected, the techniques used included: i) IFA and *gltA* gene PCR for Bartonella spp. (Norman *et al*., 1995; Sander *et al*., 2001); ii) IFA or ELISA followed by confirmatory WB of suspected or positive samples, 5S (*rrf*)-23S(*rrl*) intergenic spacer and *flgB* PCR, and isolation by direct inoculation in BSK-II medium for *B. burgdorferi* s.l. (Johnson *et al.*, 1992; Rijpkema *et al*., 1995; Lopes de Carvalho & Nuncio, 2006); iii) IFA and transposon-like repetitive region PCR for *C. burnetii* (Williems et al, 1994; Maurin & Raoult, 1999); and iv) IFA, *ompA* and *gltA* PCR, and isolation on Vero E6 shell-vials for *Rickettsia* spp. (Regnery *et al*., 1991; Bacellar *et al*., 2003; De Sousa *et al*., 2006).

Criteria for laboratory confirmation. *CEVDI/INSA*'s case definition criteria for bartonellosis, Lyme borreliosis (LB), Q fever, rickettsiosis, HGA and HME are based on published guidelines, with slight modifications in some cut-off values according to the background reactivity as determined by the National Institute of Health for the Portuguese population (Maurin & Raoult, 1999; Walker *et al*., 2000; Sander *et al*., 2001; Brouqui *et al*., 2004; Lopes de Carvalho & Nuncio, 2006; EUCALB). Thus, given an adequate epidemiologic and clinical context, a confirmed laboratory case is defined by: i) agent isolation in culture, or ii) positive PCR result with the demonstration of agent-specific DNA by subsequent amplicon sequencing, or iii) demonstration of a seroconversion or ≥four-fold increase in antibody titer between acute and convalescent samples. A single high titer also suggests disease, such as for bartonellosis and rickettsiosis (IFA IgM ≥32 and IgG ≥128), acute Q fever (IFA phase II IgM ≥50 and IgG ≥200), chronic Q fever (IFA phase I titer of IgG ≥800) and Lyme disease [IFA IgM≥32 and/or IgG≥256, Elisa IgM and/or IgG≥5, according to Virion ELISA Kit (Sirion, Germany), followed by the demonstration of 2 of 3 IgM bands and/or 5 of 10 IgG bands in western blots].

## RESULTS

From 2000 to 2006, a total of 619 sera samples from 425 patients were received by *CEVDI/INSA* for Anaplasmataceae infection diagnostic testing. In 125 patients (29.4%), more that one serum was available for testing. Moreover, all "first" blood samples received during 2006 that were collected in the proper anticoagulant were additionally used for PCR testing (n=6) and/or isolation

attempts in DH82 and HL-60 cells (n=16). In patients with Anaplasmataceae IFA-positive results, the first serum sample was also used for DNA preparation and PCR testing.

Overall, Anaplasmataceae IFA-positive reactions were detected in 5.6% (24/425) of patients, with seropositivity values ranging from 2.0% (in 2000) to 11.6% (in 2003) (Figure 1). Seropositive patients were found to react with either *A. phagocytophilum* in 70.8% (17/24) of the cases, *E. chaffeensis* in 12.5% (3/24), or with both antigens in 16.6% (4/24). However neither *A. phagocytophilum* nor *E. chaffeensis* active infections were detected by agent isolation or PCR in any sample tested during 2006. Moreover, the PCR analysis of IFA-positive sera was negative in all cases.

A detailed analysis of available data from patients that reacted only with *A. phagocytophilum* antigens revealed that almost all had polyvalent antibody titers ranging from 80 to 320 (Table 1). The only exception was patient no. 12/2005 who had a single serum sample with both IgM and IgA,G,M IFA titers of 80 and 320, respectively. Evolution of antibody titers was demonstrated among 7 of the 17 cases, showing stable IFA titers in 4 (57.1%), two-fold decreases in titers in 2 (28.6%) and seroconversion in 1 (14.3%; patient no. 11/2004).

Tests for other tick-borne agents or related bacteria conducted by *CEVDI/INSA* when requested by the physician provided laboratory evidence of bartonellosis in 1 case and acute Q fever in 5 cases. Nondiagnostic results were obtained in 11 cases. A suggestive IFA-titer of *Bartonella* sp. active infection was detected in a single serum from patient no. 2/2001 (IgM≥32 and IgG≥256, data not shown in Table 1) who also had a polyvalent *A. phagocytophilum* antibody titer of 320. Acute Q fever-compatible titers were present in 3 patients, showing either single polyvalent *A. phagocytophilum* antibody titers of 80 (patient no. 12/2004) and 160 (no. 20/2006), or a two-fold decrease in IFA-titer from 160 to 80 (no. 17/2005). Of interest is the observation that patient no. 11/2004 seroconverted to both *A. phagocytophilum* and *C. burnetii*, as evidenced by phase II IgM ≥50 and IgG ≥200 titers (data not shown in Table 1) in the second serum sample that are diagnostic of acute Q fever. Additionally, patient no. 15/2006, who presented with a stable IgA,G,M IFA-titer of 160 but was PCR-negative for *A. phagocytophilum* DNA in both buffy coat and serum, also had a seroconversion to *C. burnetii*, and a buffy coat PCR that was positive for this latter agent. Moreover, the inoculation of buffy coat into HL-60 cells resulted in the isolation of *C. burnetii*. The agent isolation was initially detected by microscopic observation of cytocentrifuged culture aliquots (Figure 2), and subsequently confirmed by using species-specific PCR (Williems *et al*., 1994). The partial sequences of transposon-like repetitive region of *C. burnetii* obtained from

this isolate are available in GenBank under the accession number EU009657. Table 1 presents all the available data from *A. phagocytophilum* IFA-positive patients detected during 2000-2006.

Among the 3 patients who had *E. chaffeensis* antibodies alone, stable IgA,G,M titers were found in two cases (patient nos. 18/2001 and 19/2002) and stable IgA,G,M and IgM titers in another case (no. 20/2005) (Table 2). In the first two patients, a seroconversion and compatible titers against *Rickettsia* spp. were found when sera were tested by IFA using *R. conorii* Malish strain antigen, supporting the laboratory diagnosis of rickettsiosis. For patient no. 20/2005 who presented with stable IgM and IgA,G,M *E. chaffeensis* titers in consecutive samples, no diagnostic laboratory tests were obtained. Moreover, the small amount of first sample serum was insufficient for DNA extraction and PCR analysis.

For four patients with both *A. phagocytophilum* and *E. chaffeensis* antibodies, stable polyvalent antibody titers (≤two-fold change) were observed (Table 3). Other laboratory tests demonstrated *C. burnetii* antibodies with titers consistent with acute (patient no. 21/2004) and chronic infections (patient no. 23/2005). In 2 other cases, differential testing was inconclusive, although patient no. 24/2006 had a stable *C. burnetii* titer, serodiagnostic criteria for active infection were not fulfilled (phase II IgM ≥50 IgG <200 and phase I IgM ≥50 IgG 400, data not shown in Table 3). Patient no. 19/2005 had the highest polyvalent titers in this study for *A. phagocytophilum* and *E. chaffeensis*, 640 and 256, respectively. Moreover he also had very high titers against *C. burnetii* (phase II IgM 200 and IgG 51,200, phase I IgM 100 and IgG 25,600; data not shown in Table 3), which in the clinical context of prolonged disease and endocarditis was highly supportive of a diagnosis of chronic Q fever.

## DISCUSSION

Since the report of *E. chaffeensis* in a Portuguese man (David de Morais *et al*., 1991), *CEVDI/INSA* has included IFA testing for this Anaplasmataceae as a routine laboratory diagnostic available for the community. In the last 6 years (2000-2006), a mean of 61 requests were received annually for *E. chaffeensis* testing, representing 3.1 to 7.4% of the total number of requests received annually for serodiagnosis at *CEVDI/INSA*. In addition, the recent detection of *A. phagocytophilum* in Portuguese *Ixodes ricinus* and *I. ventalloi* ticks (Nuncio *et al*., 2000; Santos *et al*., 2004) and the possibility of human exposure (Santos *et al*., 2006) have stimulated further

research in this field and led to the implementation in 2006 of routine laboratory diagnostic testing for this Anaplasmataceae family member. The potential for cross-reaction between *E. chaffeensis* and *A. phagocytophilum*, as previously documented in both HME and HGA case series (Bakken *et al*., 1996; Wong *et al*., 1997; Comer *et al*., 1999; Walls *et al*., 1999; Lotric-Furlan *et al*., 2006), imposes that all sera received based on a clinical suspicion of infection with either Anaplasmataceae be tested for both. Moreover, archived sera received during 2000-2005 for *E. chaffeensis* serodiagnosis were also analysed retrospectively for antibodies against *A. phagocytophilum*. This cross-testing study demonstrated a higher proportion of IFA seropositivity to *A. phagocytophilum* (17 patients) than to *E. chaffeensis* (3 patients). Reactivity to both agents was additionally found in 4 patients. These results are in accordance with those obtained in several seroprevalence studies performed on European populations (farmers, hunters, tick-exposure residents and patients with febrile illness with ascribed aetiology) and which suggest a greater exposure to *A. phagocytophilum* or an antigenically related agent, than to *E. chaffeensis* (Dumler *et al*., 1997; Thomas *et al*., 1998; Groen *et al*., 2002). In spite of *E. chaffeensis* serological reactions among patients with febrile illnesses after tick-bite, no definitive evidence of human infection has yet been obtained by either DNA amplification or agent isolation (Lotric-Furlan *et al*., 2006).

Despite the serological reactions to both *A. phagocytophilum* and *E. chaffeensis* found here, no active infections caused by either agent was definitively proven by laboratory testing. In fact, all isolation attempts using available buffy coat samples and PCR testing of IFA-positive sera for the above mentioned agents were unsuccessful. Nevertheless it is important to state that direct diagnosis suffered from constraints that could have influenced the final results. For example, isolations in both HL-60 and DH82 cells were only routinely attempted in 2006. Moreover, for the great majority of seroreactive patients, an adequate whole-blood sample for direct testing was not provided. Even using known sensitive PCR approaches, the ability to amplify small amounts of target DNA (Sumner *et al*., 1997; Massung *et al*., 1998; Caspersen *et al*., 2002) from archived sera of known positive patients is only 14% sensitive, a significant limitation to this approach (Comer *et al*., 1999). In addition, very little information about antecedent treatment that could reduce Anaplasmataceae DNA was available, further confounding detection methods. Thus, despite negative results in both culture and sensitive molecular detection methods, the possibility of false-negative results can not be excluded.

Despite the lack of direct confirmation of Anaplasmataceae active human infections, one patient seroconverted to *A. phagocytophilum* antigen in consecutive samples, fulfilling serological,

criteria for a confirmed case. However, when tested for other tick-borne or related agents by physician request and presumptive diagnosis, one patient also seroconverted to *C. burnetii* developing an antibody titer in the second serum that was alone highly presumptively diagnostic for acute Q fever. Additionally, other patients with serological reactivity to *A. phagocytophilum* alone or in association with *E. chaffeensis* also had laboratory diagnostic results compatible for Q fever in 6 cases and for bartonellosis in 1 case. Of interest was the direct confirmation of Q fever in 1 patient by isolation of *C. burnetii* in HL-60 cells and by PCR detection of *C. burnetii* DNA in buffy coat and culture samples. The HL-60 cells are currently known as an *in vitro* cellular target for *A. phagocytophilum* cultivation, due to its tropism for polymorphonuclear leucocytes. These cells are derived from a patient with promyelocytic leukaemia, but they can also be induced to terminal differentiation into either monocyte/macrophage-like cells or neutrophil-like cells by stimuli such all-trans Retinoic acid (granulocyte differentiation) or Vitamin D3 (monocyte/macrophage differentiation), and even long-term culture. The fact that our cultures were maintained ≥ 1 month after inoculation might have led to HL-60 monocyte/macrophage differentiation, as this agent is known to preferentially target cells from the mononuclear lineage.

The possibility of cross-reactivity between non-related agents has been already addressed. Brouqui and coworkers (2001), in an evaluation trial of serodiagnosis by IFA testing using distinct *A. phagocytophilum* from different sources as well as varying antigen preparations demonstrated cross-reactions in patients with confirmed Q fever and bartonellosis. In the present series, Q fever was the main disease identified in cross-reactions with *A. phagocytophilum*, although the results of an internal *CEVDI/INSA* study performed using serum from confirmed *C. burnetii* cases demonstrated that not all patients present with Anaplasmataceae cross-reactive samples (data not shown). Whether these findings represent a distinct cross-reactive potential for different *C. burnetii* strains that is directed toward common antigens such as heat shock proteins, distinct host immune responses to infections, or antibody induced by active dual or past exposures to both *A. phagocytophilum* and *C. burnetii*, remains to be ascertained.

In Portugal, Q fever was recognized in 1948 (Fonseca *et al*., 1949 in Santos *et al*., 2007), and has been listed as a reportable disease since 1999 (http://www.dgsaude.pt). On average, 0.14 cases per $10^5$ inhabitants are reported annually (1999-2003), however official reports may not accurately reflect the true Q fever incidence and little information is available regarding *C. burnetii* natural history in Portugal (Santos *et al*., 2007). Domestic mammals, especially cattle, sheep, goats, dogs and cats are regarded as the most important reservoirs for human infection, and the

pathogen is usually acquired by aerosol contamination (Maurin and Raoult, 1999). Although tick-exposure is not considered relevant in the epidemiology of Q fever human cases, ticks are thought to play an important role in the maintenance of agent's enzootic cycle among wild animals (Maurin & Raoult, 1999). Several tick species have been found infected with *C. burnetii* (Maurin & Raoult, 1999) and the isolation of this agent from *I. ricinus* has been reported in Europe (Rehacek *et al*., 1994; Jacomo *et al*., 2002). Moreover, *A. phagocytophilum* coinfections with *Bartonella henselae* and *Babesia* spp. have also been sporadically reported in *Ixodes* ticks (Adelson *et al*., 2004; Holden *et al*., 2006). Thus, like other *Ixodes*-borne agents that share vector ticks, such as *B. burgdorferi s.l.*, *A. phagocytophilum* and *C. burnetii* or other agent's enzootic cycles might potentially overlap. A previous study of Portuguese patients with confirmed and suspected LB showed a significant association of with *A. phagocytophilum* seropositivity when comparing to controls (Santos *et al*., 2006). The additional identification of *A. phagocytophilum* seroreactivity among patients with confirmed Q fever and the fact that *I. ricinus* is one of the most frequently reported tick species parasitizing humans in Portugal, underscores the need for further investigation regarding the potential exposure of humans to several *Ixodes*-borne agents in Portugal.

Another interesting finding of this work was the detection of antibody titers only to *E. chaffeensis* antigen in 2 patients who presented with compatible laboratory diagnostics for rickettsiosis. Thus far, the apparent lack of any definitive *E. chaffeensis* identification by DNA detection or agent isolation in most European countries, as in Portugal, suggests exposure to other *Ehrlichia* spp. as a possible explanation for the serological reactions. *Rhipicephalus sanguineus*, one of the most prevalent tick species in Portugal, is a recognized vector of the two *Rickettsia conorii* strains causing Mediterranean spotted fever (MSF) (Bacellar, 1999; Santos-Silva *et al*., 2006). This disease is presently the most important rickettsial infection in Portugal due not only to its high reported incidence and associated fatality rates, but also because of the broad-geographic distribution of cases throughout Portugal (Amaro *et al*., 2003; De Sousa *et al*., 2003). Additionally, *R. sanguineus* is a recognized vector of the canine Anaplasmataceae pathogen, *Ehrlichia canis*. Canine disease due to *E. canis* infection has long been suggested by several serological studies and was definitively proven by a PCR-based study of a clinically-ill Portuguese dog population (Alexandre *et al*., in submission). Thus, the likely contact of *R. sanguineus* with humans and their potential for harbouring an *Ehrlichia* sp. might belie serological reactions detected using antigens of the closely related *E. chaffeensis* given the absence of irrefutable proof for its presence. In fact, *E.*

*canis* can evoke immune responses in Venezuelian patients with both asymptomatic and symptomatic infections (Perez *et al*., 1996, 2006). Thus serological cross-reactions after *E. canis* exposure in humans could be considered as a possible explanation for antibodies reactiveness with *E. chaffeensis*, although the human pathogenic potential of this canine agent deserves considerable further investigation.

In conclusion, although no definitively confirmed Anaplasmataceae infection was detected in the present study it is suggested that in Portuguese patients with a clinical suspicion of tick-borne disease or related illness, exposure to *A. phagocytophilum*, or a close related agent, is more likely to occur than to *E. chaffeensis*, or other *Ehrlichia* spp. Moreover, serological reactions to *A. phagocytophilum* antigen in patients with confirmed Q fever and to *E. chaffeensis* in patients with rickettsiosis were recognized. Whether these findings reflect serological cross-reactions, coinfections, or present/past exposure deserves further investigation. A continuing integrated analysis of Anaplasmataceae and other tick-borne or related agent laboratory diagnostics, when justified by clinical suspicion, is indispensable to better understand the present results. Moreover, the potential for native Anaplasmataceae infections in residents at risk for tick-borne disease, especially due to *A. phagocytophilum*, or imported cases associated with international travelers to European- or US-endemic areas, suggests the need for continuing active surveillance.

## ACKNOWLEDGMENTS

This research was partially supported by the Portuguese government through the *Fundação para a Ciência e a Tecnologia* grant BD/8610/2002. A special thanks to Teresa Luz and Paulo Parreira *CEVD/INSA*'s technicians that are directly involved in the serodiagnosis.

## REFERENCES

1. ADELSON ME, RAO RV, TILTON RC, CABETS K, ESKOW E, FEIN L, OCCI JL, MORDECHAI E. Prevalence of *Borrelia burgdorferi*, *Bartonella* spp., *Babesia microti*, and *Anaplasma phagocytophila* in *Ixodes scapularis* ticks collected in Northern New Jersey. J Clin Microbiol. 2004;42(6):2799-801

2. ALEXANDRE N, SANTOS AS, NÚNCIO MS, SOUSA S, BOINAS F, BACELLAR F. First molecular detection of *Ehrlichia canis* in dog, Portugal. In submission.

3. AMARO M, BACELLAR F, FRANÇA A. Report of eight cases of fatal and severe Mediterranean spotted fever in Portugal. Ann NY Acad Sci. 2003;990:331-43.

4. ANDERSON BE, DAWSON JE, JONES DC, WILSON KH. *Ehrlichia chaffeensis*, a new species associated with human ehrlichiosis. J Clin Microbiol. 1991;29(12):2838-42.

5. BACELLAR FC. Ticks and Spotted Fever Rickettsiae in Portugal. *In*: Rickettsiae and Rickettsial Diseases at the Turn of the Third Millenium. Raoult D, Brouqui P, editors. Paris: Elsevier; 1999. p. 423-7.

6. BACELLAR F, SOUSA R, SANTOS A, SANTOS-SILVA M, PAROLA P. Boutonneuse fever in Portugal: 1995-2000. Data of a state laboratory. Eur J Epidemiol. 2003;18:275-7.

7. BAKKEN JS, DUMLER JS, CHEN SM, ECKMAN MR, VAN ETTA LL, WALKER DH. Human granulocytic ehrlichiosis in the upper Midwest United States: a new species emerging? JAMA. 1994;272:212–8.

8. BAKKEN JS, KRUETH J, WILSON-NORDSKOG C, TILDEN RL, ASANOVICH K, DUMLER JS. Clinical and laboratory characteristics of human granulocytic ehrlichiosis. JAMA. 1996;275:199-205.

9. BROUQUI P, BACELLAR F, BARANTON G, BIRTLES RJ, BJOERSDORFF A, BLANCO JR, CARUSO G, CINCO M, FOURNIER PE, FRANCAVILLA E, JENSENIUS M, KAZAR J, LAFERL H, LAKOS A, LOTRIC FURLAN S, MAURIN M, OTEO JA, PAROLA P, PEREZ-EID C, PETER O, POSTIC D, RAOULT D, TELLEZ A, TSELENTIS Y, WILSKE B; ESCMID Study Group on *Coxiella*, *Anaplasma*, *Rickettsia* and *Bartonella*; European Network for Surveillance of Tick-Borne Diseases. Guidelines for the diagnosis of tick-borne bacterial diseases in Europe. Clin Microbiol Infect. 2004;10(12):1108-32.

10. BROUQUI P, SALVO E, DUMLER JS, RAOULT D. Diagnosis of granulocytic ehrlichiosis in humans by immunofluorescence assay. Clin Diagn Lab Immunol. 2001;8(1):199-202.

11. BULLER RS, ARENS M, HMIEL SP, PADDOCK CD, SUMNER JW, RIKHISA Y, UNVER A, GAUDREAULT-KEENER M, MANIAN FA, LIDDELL AM, SCHMULEWITZ N, STORCH GA. *Ehrlichia ewingii*, a newly recognized agent of human ehrlichiosis. N Engl J Med. 1999;341(3):148-55.

12. CASPERSEN K, PARK J-H, PATIL S, DUMLER JS. Genetic variability and stability of *Anaplasma phagocytophila msp2* (*p44*). Infect Immun. 2002;70(3):1230-4.

13. CHEN S-M, DUMLER JS, BAKKEN JS, WALKER DH. Identification of a granulocytotropic ehrlichia species as the etiologic agent of human diseases. J Clin Microbiol. 1994;32(3):589-95.

14. COMER JA, NICHOLSON WL, OLSON JG, CHILDS JE. Serologic testing for human granulocytic ehrlichiosis at a National Referral Center. J Clin Microbiol. 1999;37(3):558-64.

15. DAVID DE MORAIS JD, DAWSON JE, GREENE C, FILIPE AR, GALHARDAS LC, BACELLAR F. First European cases of ehrlichiosis. Lancet. 1991; 338:633–4.

16. DAWSON JE, ANDERSON BE, FISHBEIN DB, SANCHEZ JL, GOLDSMITH CS, WILSON KH, DUNTLEY CW. Isolation and characterization of an *Ehrlichia* sp. from a patient diagnosed with human ehrlichiosis. J Clin Microbiol. 1991;29(12):2741-5.

17. DE SOUSA R, BARATA C, VITORINO L, SANTOS-SILVA M, CARRAPATO C, TORGAL J, WALKER D, BACELLAR F. *Rickettsia sibirica* isolation from a patient and detection in ticks, Portugal. Emerg Infect Dis. 2006;12:1103-8.

18. DE SOUSA R, NOBREGA SD, BACELLAR F, TORGAL J. Mediterranean spotted fever in Portugal: risk factors for fatal outcome in 105 hospitalized patients. Ann NY Acad Sci. 2003;990:285-94.

19. DUMLER JS, BARBET AF, BEKKER CPJ, DASCH GA, PALMER GH, RAY SC, RIKIHISA Y, RURANGIRWA FR. Reorganization of genera in the families Rickettsiaceae and Anaplasmataceae in the order Rickettsiales: unification of some species of *Ehrlichia* with *Anaplasma*, *Cowdria* with *Ehrlichia* and *Ehrlichia* with *Neorickettsia*, description of six new species combinations and designation of *Ehrlichia equi* and 'HGE agent' as subjective synonyms of *Ehrlichia phagocytophila*. Int J Syst Evol Microbiol. 2001;51:2145-65.

20. DUMLER JS, DOTEVALL L, GUSTAFSON R, GRANSTROM M. A population- based seroepidemiologic study of human granulocytic ehrlichiosis and Lyme borreliosis on the west coast of Sweden. J Infect Dis. 1997;175:720-2.

21. DUMLER JS, RIKIHISA Y, DASCH G. Genus I. *Anaplasma* Theiler 1910, 7 AL emend. Dumler, Barbet, Bekker, Dasch, Palmer, Ray, Rikihisa and Rurangirwa 2001, 2157. In: Bergey's Manual of Systematic Bacteriology. Garrity G, Brenner DJ, Krieg NR, Staley JR, editors. Volume II The Proteobacteria Part C The Alpha-, Beta-, Delta-, and Epsilonproteobacteria. 2nd edition. New-York: Spriger; 2005b. p. 117-25.

22. EUCALB- European Union Concerted Action on Lyme Borreliosis [updated 2006]. Available from: http://www.oeghmp.at/eucalb/diagnosis_clinical-features-ds.html.

23. GOODMAN JL, NELSON C, VITALE B, MADIGAN JE, DUMLER JS, KURTTI TJ, MUNDERLOH UG. Direct cultivation of the causative agent of human granulocytic ehrlichiosis. N Engl J Med. 1996;334(4):209–15.

24. GROEN J, KORAKA P, NUR YA, AVSIC-ZUPANC T, GOESSENS WH, OTT A, OSTERHAUS AD. Serologic evidence of ehrlichiosis among humans and wild animals in The Netherlands. Eur J Clin Microbiol Infect Dis. 2002;21(1):46-9.

25. HOLDEN K, BOOTHBY JT, KASTEN RW, CHOMEL BB. Co-detection of *Bartonella henselae*, *Borrelia burgdorferi*, and *Anaplasma phagocytophilum* in *Ixodes pacificus* ticks from California, USA. Vector Borne Zoonotic Dis. 2006;6(1):99-102.

26. JACOMO V, KELLY PJ, RAOULT D. Natural history of *Bartonella* infections (an exception to Koch's postulate). Clin Diagn Lab Immunol. 2002;9(1):8-18.

27. JOHNSON BJ, HAPP CM, MAYER LW, PIESMAN J. Detection of *Borrelia burgdorferi* in ticks by species-specific amplification gene. Am J Trop Med Hyg. 1992;47(6):730-41.

28. LOPES DE CARVALHO I, NÚNCIO MS. Laboratory diagnosis of Lyme borreliosis in Portuguese National Institute of Heath (1990-2004). Eurosurveillance. 2006;11(10):257-260.

29. LOTRIC-FURLAN S, ROJKO T, PETROVEC M, AVSIC-ZUPANC T, STRLE F. Epidemiological, clinical and laboratory characteristics of patients with human granulocytic anaplasmosis in Slovenia. Wien Klin Wochenschr. 2006;118(21-22):708-13.

30. MAEDA K, MARKOWITZ N, HAWLEY RC, RISTIC M, COX D, MCDADE JE. Human infection with *Ehrlichia canis*, a leukocytic rickettsia. N Eng J Med. 1987;316:853-6.

31. MASSUNG RF, SLATER K, OWENS JH, NICHOLSON WL, MATHER TN, SOLBERG VB, OLSON JG. Nested PCR assay for detection of granulocytic ehrlichiae. J Clin Microbiol. 1998;36(4):1090-5.

32. MAURIN M, RAOULT, D. Q Fever. Clin Microbiol Rev. 1999;12(4):518-53.

33. MISAO T, KOBAYASHI Y. Studies on infectious mononucleosis. I. isolation of etiologic agent from blood, bone marrow and lymph node of a patient with infectious mononucleosis by using mice. Kyushu J Med Sci. 1955;6:145-52.

34. NORMAN AF, REGNERY R, JAMESON P, GREENE C, KRAUSE DC. Differentiation of *Bartonella*-like isolates at the species level by PCR-restriction fragment length polymorphism in the citrate synthase gene. J Clin Microbiol. 1995;33(7):1797-803.

35. NÚNCIO MS, SCHOULS LM, VAN DE POL I, BACELLAR F, FILIPE A. The role of *Ixodes ricinus* as a vector of zoonotic agents in Madeira Island. In: Proceedings of the Seventh Bienal Meeting of European Tick Study Group, Oxford, United Kingdom, 2000 April 28.

36. PEREZ, M, RIKIHISA, Y, WEN, B. *Ehrlichia canis*-like agent isolated from a man in Venezuela: antigenic and genetic characterization. J Clin Microbiol. 1996;34:2133-9.

37. PEREZ M., BODOR M., ZHANG C., XIONG Q., RIKIHISA Y. Human infection with *Ehrlichia canis* accompanied by clinical signs in Venezuela. Ann NY Acad Sci. 2006;1078:110-7.

38. REGNERY, RL, SPRUILL CL, PLIKAYTIS BD. Genotypic identification of rickettsiae and estimation of intraspecies sequences divergence for portions of two rickettsial genes. J Bacteriol. 1991;173(5):1576-89.

39. REHACEK J, KAASERER B, URVOLGYI J, LUKACOVA M, KOVACOVA E, KOCIANOVA E. Isolation of *Coxiella burnetii* and of an unknown rickettsial organism from *Ixodes ricinus* ticks collected in Austria. Eur J Epidemiol. 1994;10(6):719-23.

40. RIJPKEMA SG, MOLKENBOER MJ, SCHOULS LM, JONGEJAN F, SCHELLENKENS JF. Simultaneous Detection and genotyping of three genomic groups of *Borrelia burgdorferi sensu lato* in Dutch *Ixodes ricinus* Ticks by characterization of the Amplified Intergenic Spacer region between 5S and 23S rRNA Genes. J Clin Microbiol. 1995;33(12):3091-5.

41. SANDER A, BERNER R, RUESS M. Serodiagnosis of cat scratch disease: response to *Bartonella heneslae* in children and a review of diagnostic methods. Eur J Clin Microbiol Infect Dis. 2001;20:392-401.

42. SANTOS AS, AMARO F, SANTOS-SILVA M, SOUSA R, MATHIAS ML, RAMALHINHO MG, NUNCIO MS, ALVES MJ, BACELLAR F; DUMLER JS. Detection of antibodies against *Anaplasma phagocytophilum* in wild-rodents, Portugal. In submission.

43. SANTOS AS, BACELLAR F, DUMLER JS. Human exposure to *Anaplasma phagocytophilum* in Portugal. Ann NY Acad Sci. 2006;1078:100-5.

44. SANTOS AS, BACELAR F, FRANÇA A. [Q fever: a revision of concepts]. Rev Port Med Int 2007;14(2):90-9. [Article in Portuguese and abstract in English]

45. SANTOS AS, SANTOS-SILVA MM, ALMEIDA VC, BACELLAR F, DUMLER JS. Detection of *Anaplasma phagocytophilum* DNA in *Ixodes* ticks (Acari: Ixodidae) from Madeira Island and Setubal District, mainland Portugal. Emerg Infect Dis. 2004;10(9):1643-8.

46. SANTOS-SILVA M, SOUSA R, SANTOS AS, LOPES D, QUEIJO E, DORETA A, VITORINO L, BACELLAR F. Ticks and tick-borne rickettsiae surveillance in Montesinho Natural Park – Portugal. Ann NY Acad Sci. 2006;1078:137-42.

47. SUMNER JW, NICHOLSON WL, MASSUNG RF. PCR amplification and comparison of nucleotide sequences from the *groESL* heat shock operon of *Ehrlichia* species. J Clin Microbiol. 1997;35(8):2087-92.

48. THOMAS DR, SILLIS M, COLEMAN TJ, KENCH SM, OGDEN NH, SALMON RL, MORGAN-CAPNER P, SOFTLEY P, MEADOWS D. Low rates of ehrlichiosis and Lyme borreliosis in English farmworker. Epidemiol Infect. 1998;12:609-14.

49. WALKER DH, BAKKEN J, BROUQUI P, CHILDS J, CULLMAN L, DAWSON J, DUMLER S, FINGERLE V, GOODMAN J, HOGAN P, HOGREFE W, LIZ J, MAGNARELLI LA, MARKLEY J, MASSUNG R, MASTERS EJ, MURPHY C, OLANO J, PADDOCK C, PETROVEC M, RIKIHISA Y, STANDAERT S, WORMSER GP, YU X-J, AVSIC-ZUPANC T. Diagnosing human ehrlichioses: current status and recommendations. ASM News. 2000;66(5):287-90.

50. WALLS JJ, AGUERO-ROSENFELD M, BAKKEN JS, GOODMAN JL, HOSSAIN D, JOHNSON RC, DUMLER JS. Inter- and intralaboratory comparison of *Ehrlichia equi* and human granulocytic ehrlichiosis (HGE) agent strains for serodiagnosis of HGE by the immunofluorescent-antibody test. J Clin Microbiol. 1999;37(9):2968-73.

51. WILLIEMS H, THIELE D, FRÖLICH-RITTER R, KRAUSS H. Detection of *Coxiella burnetii* in Cow's milk using the polymerase chain reaction (PCR). J Vet Med B. 1994;41:580-7.

52. WONG SJ, BRADY GS, DUMLER JS. Serological responses to *Ehrlichia equi*, *Ehrlichia chaffeensis*, and *Borrelia burgdorferi* in patients from New York State. J Clin Microbiol. 1997;35(9):2198-205.

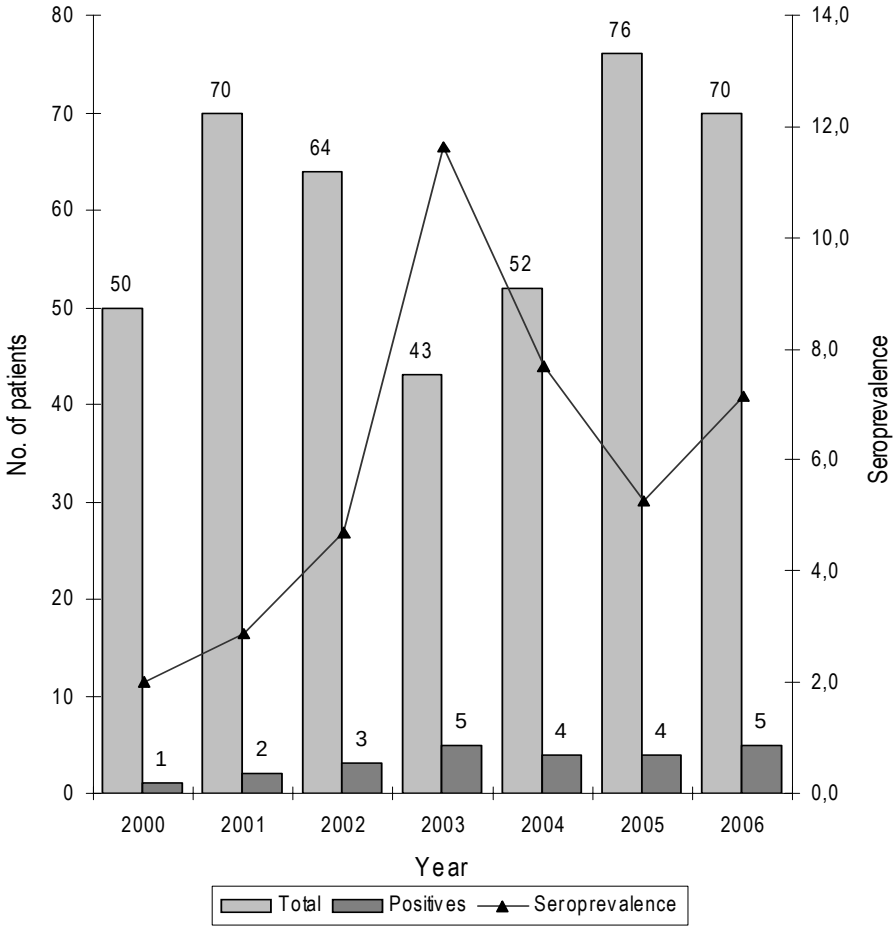



FIGURE 1. Number of patients that had at least one sera sample submitted to *CEVDI/INSA* with a request for *A. phagocytophilum* and/or *E. chaffeensis* diagnosis during 2000-2006.

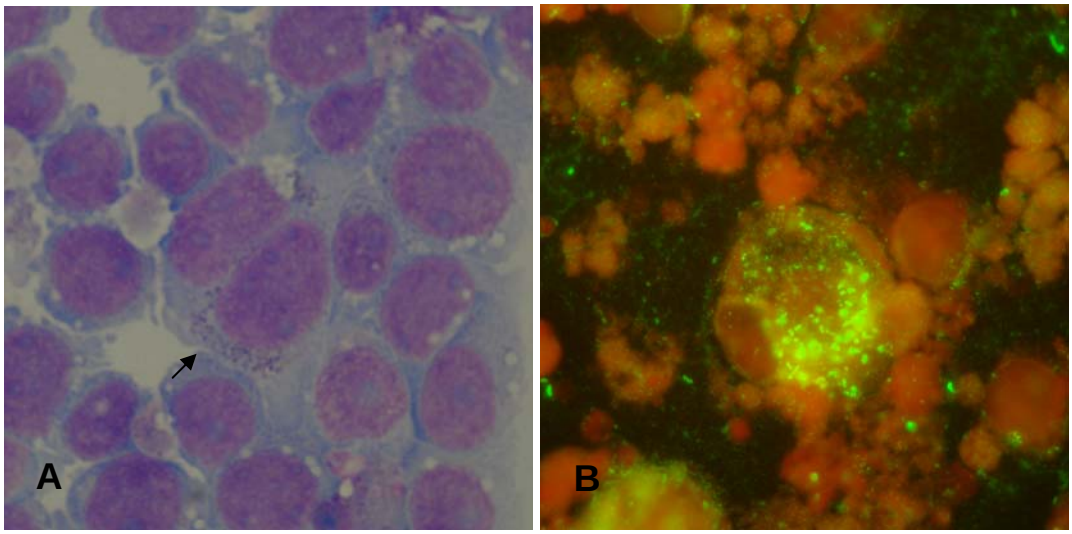



FIGURE 2. Light microscopic appearance of *C. burnetti* human isolate in HL-60 cells. Culture cytocentrifuge preparations stained by Diff-Quik (A) and by IFA, using a positive control sera (B). Arrow indicate intracytoplasmic inclusions filled with numerous bacteria, 1000X magnification.

TABLE 1. Patients with positive results for *A. phagocytophilum* in serologic tests (2000-2006).

| Patient No./ Year | Gender/Age(y)/ Residence[a] | Clinical information[b] | Sample date | *A. phagocytophilum* | | | Other pathogen tests[c] | Interpretation of laboratory diagnosis |
|---|---|---|---|---|---|---|---|---|
| | | | | IFA (IgM/IgA,M,G) | PCR (Blood/Sera) | Isolation (HL60) | | |
| 1/2000 | M/52/Lisboa | Febrile illness | 06/Jul | –/80 | NA/– | NA | Ec (S –) | Inconclusive |
| 2/2001 | F/NA/Lisboa | Febrile illness | 19/Jun | –/320 | NA/– | NA | Ba (S +); Ec, Rk (S –) | Bartonellosis (compatible titers) |
| 3/2002 | F/NA/Santarém | Febrile illness | 04/Apr | –/160 | NA/– | NA | Ec, Rk (S –) | Inconclusive |
| 4/2002 | M/43/Setúbal | NA | 12/Aug | –/80 | NA/NA | NA | Ec, Rk (S –) | Inconclusive |
| 5/2003 | M/35/Lisboa | NA | 21/Jun | –/80 | NA/– | NA | Ec, Rk (S –) | Inconclusive |
| 6/2003 | M/64/Santarém | Febrile illness | 02/Jun<br>09/Oct | –/80<br>–/80 | NA/– | NA | Ec, Rk (S –)<br>Ec, Rk (S –) | Inconclusive |
| 7/2003 | M/44/Lisboa | Polyarthralgia | 13/Mar | –/80 | NA/– | NA | Ec, Rk (S –) | Inconclusive |
| 8/2003 | M/49/Évora | Febrile illness | 10/Apr<br>17/Apr<br>23/Sep | –/80<br>–/80<br>–/80 | NA/– | NA | Ec, Rk (S –)<br>Ec, Rk (S –)<br>Cb; Ec, Rk (S –) | Inconclusive |
| 9/2003 | M/40/Évora | Arthritis | 26/Dec<br>19/Feb | –/160<br>–/80 | NA/– | NA | Ec, Rk (S –)<br>Bb, Ec, Rk (S –) | Inconclusive |
| 10/ 2004 | M/NA/Évora | NA | 05/Nov | –/80 | NA/– | NA | Cb (S +); Ec, Rk (S –) | Acute Q fever (compatible titers) |

TABLE 1 (cont.). Patients with positive results for *A. phagocytophilum* in serologic tests (2000-2006).

| Patient No./ Year | Gender/Age(y)/ Residence[a] | Clinical information[b] | Sample date | *A. phagocytophilum* | | | Other pathogen tests[c] | Interpretation of laboratory diagnosis |
|---|---|---|---|---|---|---|---|---|
| | | | | IFA (IgM/IgA,M,G) | PCR (Blood/Sera) | Isolation (HL60) | | |
| 11/2004 | M/NA/Setúbal | Febrile illness | 16/May<br>18/Jul | –/–<br>–/80 | NA/– | NA | Cb, Ec (S –); Rk (S/P/I –)<br>Cb (S +); Ec, Rk (S –) | Acute Q fever (seroconversion); |
| 12/2005 | M/60/Évora | Polyarthralgia | 09/Jun | 80/320 | NA/– | NA | Cb, Ec (S –); Rk (S/P/I –) | Inconclusive |
| 13/2005 | F/51/Lisboa | Febrile illness | 06/Feb<br>25/Feb | –/160<br>–/80 | NA/– | NA | Cb (S +); Ec, Rk (S –)<br>Cb (S +); Ec, Rk (S –) | Acute Q fever (compatible titers) |
| 14/2006 | F/68/Évora | Febrile illness | 13/Mar<br>03/Apr | NA<br>–/160 | NA/NA | NA | Ec, Rk (S –)<br>Cb (S +); Ec, Rk (S –) | Acute Q fever (compatible titers) |
| 15/2006 | F/39/Faro | Febrile illness | 27/Jun<br>03/Jul | –/160<br>–/160 | –/– | – | Cb (S –; P +); Bb (S ––); Ec, Rk (S/P/I –)<br>Cb (S +); Bb, Ec, Rk (S –) | Acute Q fever (seroconversion; *C. burnetii* isolation on HL60; DNA detection in both culture, and buffy coat) |
| 16/2006 | F/53/Coimbra | Febrile illness | 14/Nov<br>30/Nov | –/80<br>–/80 | NA– | NA | Ba; Cb, Ec, Rk (S –)<br>Ba; Cb, Ec, Rk (S –) | Inconclusive |
| 17/2006 | F/NA/NA | Sepsis | 29/Nov | –/80 | –/– | – | Bb, Cb, Ec, Rk (S/P –) | Inconclusive |

(-) Negative result; (+) Positive result; (NA) Not available sample; [a]District of residence; [b]Clinical information available in submission forms; [c]Available information regarding other tick-borne agents or related bacteria tested in *CEVDI/INSA* according to physician suspicion - (Bb) *Borrelia burgdorferi s.l.,* (Cb) *Coxiella burnetii*, (Ba) *Bartonella* spp., (Ec) *Ehrlichia chaffeensis*, (Rk) *Rickettsia* spp. The laboratory diagnosis was based in (S) serology, (P) PCR testing and (I) isolation attempts.

TABLE 2. Patients with positive results for *E. chaffeensis* in serologic tests (2000-2006).

| Patient No./ Year | Gender/Age(y)/ Residence[a] | Clinical information[b] | Sample date | *E. chaffeensis* | | | Other pathogen tests[c] | Interpretation of laboratory diagnosis |
|---|---|---|---|---|---|---|---|---|
| | | | | IFA (IgM/ IgA,M,G) | PCR (Blood/Sera) | Isolation (DH82) | | |
| 18/2001 | F/NA/Évora | NA | 17/Aug | –/64 | NA/– | NA | Ap, Rk (S –) | Rickettsiosis (seroconversion) |
| | | | 18/Oct | –/64 | | | Ap (S -); Rk (S +) | |
| 19/2002 | F/NA/Évora | Febrile illness | 20/Sep | –/64 | NA/– | NA | Ap (S -); Rk (S +) | Rickettsiosis (compatible titers) |
| | | | 18/Oct | –/64 | | | Ap (S -); Rk (S +) | |
| 20/2005 | F/29/Évora | Febrile illness | 30/Jun | 128/128 | NA/NA | NA | Ap, Cb (S –); Rk (S/P/I –) | Inconclusive |
| | | | 21/ Jul | 128/128 | | | Ap, Cb, Rk (S –) | |

(-) Negative result; (+) Positive result; (NA) Not available sample; [a]District of residence; [b]Clinical information available in submission forms; [c]Available information regarding other tick-borne agents or related bacteria tested in *CEVDI/INSA* according to physician suspicion – (Ap) *Anaplasma phagocytophilum*, (Bb) *Borrelia burgdorferi s.l.,* (Cb) *Coxiella burnetii*, (Ba) *Bartonella* spp., (Rk) *Rickettsia* spp. The laboratory diagnosis was based in (S) serology, (P) PCR testing and (I) isolation attempts.

TABLE 3. Patients with positive results for both *A. phagocytophilum* and *E. chaffeensis* in serologic tests (2000-2006).

| Patient No./ Year | Gender/Age(y)/ Residence[a] | Clinical information[b] | Sample date | Anaplamataceae | *A. phagocytophilum* | | *E. chaffeensis* | | Other pathogen tests[c] | Interpretation of laboratory diagnosis |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | PCR (Blood/Sera) | IFA (IgM/ IgA,M,G) | Isolation (HL60) | IFA (IgM/ IgA,M,G) | Isolation (DH82) | | |
| 21/2004 | F/60/Évora | Febrile illness with rash | 25/Mar | NA/NA | NA | NA | NA | NA | Rk (S –) | Acute Q fever |
| | | | 30/Mar | | –/80 | | –/64 | | Cb (S +); Rk (S –) | (compatible titers) |
| | | | 06/May | | –/80 | | –/128 | | Cb (S +); Rk (S –) | |
| 22/2004 | M/33/Lisboa | NA | 02/Jun | NA/NA | NA | NA | –/128 | NA | Cb (S –); Rk (+) | Inconclusive |
| | | | 13/Jun | | –/160 | | –/64 | | | |
| 23/2005 | M/52/Évora | Endocarditis | 23/Aug | NA/– | –/640 | NA | –/256 | NA | Cb (S +); Rk (S –) | Chronic Q fever |
| | | | 08/Sep | | –/320 | | –/128 | | Cb (S +); Rk (S –) | (compatible titers) |
| | | | 29/Sep | | –/320 | | –/128 | | Cb (S +); Rk (S –) | |
| | | | 13/Dec | | –/320 | | –/128 | | Cb (S +) | |
| 24/2006 | M/58/Évora | NA | 19/Dec | –/– | –/640 | – | –/128 | – | Cb (S +); Rk (S/I –) | Inconclusive |

(-) Negative result; (+) Positive result; (NA) Not available sample; [a]District of residence; [b]Clinical information available in submission forms; [c]Available information regarding other tick-borne agents or related bacteria tested in *CEVDI/INSA* according to physician suspicion - (Cb) *Coxiella burnetii*, (Rk) *Rickettsia* spp. The laboratory diagnosis was based in (S) serology, (P) PCR testing and (I) isolation attempts.

# CHAPTER VI

## FINAL REMARKS AND FUTURE PERSPECTIVES

# FINAL REMARKS AND FUTURE PERSPECTIVES

This thesis continues earlier studies on *Ehrlichia* species infections started at *Centro de Estudos de Vectores e Doenças Infecciosas*, *Instituto Nacional de Saúde Dr. Ricardo Jorge* (*CEVDI/INSA*) during the 1990s. It focuses on methodological training, especially regarding *A. phagocytophilum*. Much of the work was under the guidance of Professor John Stephen Dumler, initially at the Unité des Rickettsies - Faculté de Medécine, Marseille - France, and later continued in the Division of Medical Microbiology, Department of Pathology, The Johns Hopkins University School of Medicine, Baltimore, Maryland - USA. As presented in Chapter II, the technical training included the integration of research teams resulting in broad studies of Anaplasmataceae, such as "Distribution of "*Ehrlichia walkeri*" in *Ixodes ricinus* (Acari:Ixodidae) from the Northern Part of Italy", conducted while training at the Unité des Rickettsies. The opportunity to work and learn in Research Units devoted to Anaplasmataceae was essential for the proposed objectives of this thesis, allowing technology transfer and development of this area research investigation at *CEVDI/INSA*. Establishing the foundations for a concerted study on *A. phagocytophilum* enabled cooperation in a Spanish study partially developed by our laboratory "Detection of a non-pathogenic variant of *Anaplasma phagocytophilum* in *Ixodes ricinus* from La Rioja, Spain", and permitted the fundamental study of *A. phagocytophilum* in Portugal, presented in Chapters III to V either as published articles, or as original studies submitted for publication in scientific journals.

The study of vector ticks potentially involved in *A. phagocytophilum* enzootic cycles, addressed in Chapter III, was based on collaborative field-work developed in coordination with other research teams of *CEVDI/INSA* in order to promote a fuller understanding of ticks and tick-borne agents of potential Public Health relevance in Portugal. Several regions of the country were selected for the field-trials, either because *Ixodes* species were known to be present, or because specific interest in a given tick-borne disease was expressed for integration with ongoing projects. Nevertheless, it is important to state that several areas of interest were not covered by this study due to time restrictions or high cost usually associated with this kind of research.

The majority of ticks included in the present study were obtained by flagging vegetation in selected collection sites. Additional specimens were also collected from parasitized domestic and

wild animals, as well as from humans. The analysis of questing ticks generally allows a better assessment of the tick-borne agent's prevalence in a given environment, by reducing the potential bias introduced by infected host blood resulting in positive results when vector competence for pathogen replication and transmission is uncertain. Moreover, the study of free-living ticks has additional informative value with regard to the potential of a given species to sustain the agent transstadially, which is one of the fundamental requirements considered for competence as a vector. Regardless, important information can also be obtained with the analysis of ticks in their parasitic phase, especially regarding the potential role of wild and domestic animals as feeding-support for hematophagous arthropods of interest and their involvement in an infectious agent's natural cycles when serving as either susceptible vertebrate host or reservoir.

One conclusion of this study was that *A. phagocytophilum* persists on Madeira Island, Madeira Archipelago. The presence of this agent was first reported in questing *I. ricinus* collected on the Island by Núncio and coworkers (2000) and observed again in field work performed during this study. The estimated prevalence of *A. phagocytophilum* on Madeira Island ticks was 4 - 7.5%, which is in the range of 0 – 57.1% described in questing *I. ricinus* from other European countries, as detailed in Chapter 1 - State of the Art. Studies using a greater number of samples, and targeting different seasons, locations and habitats were identified as important requirements for confirming levels of infection and to better investigate the natural cycles of the agent on the Island. Two projects have already been submitted to obtain government financial support to continue research on Madeira Island. Another interesting finding was the detection of a natural focus of *A. phagocytophilum* that involves other *Ixodes* species - *I. ventalloi* in Baixa de Palmela (Setúbal District, mainland). Infected ticks were obtained from both vegetation and a stray cat (*Felis catus domesticus*). This was the first documentation of *A. phagocytophilum* infection in *I. ventalloi* ticks. Moreover, it added domestic cats to the national list of vertebrate hosts parasitized by this tick species and potentially involved in *A. phagocytophilum* natural cycles. The highlighted findings are presented in the article "Detection of *Anaplasma phagocytophilum* DNA in *Ixodes* ticks (Acari:Ixodidae) from Madeira Island and Setúbal District, Mainland Portugal". This association is now reinforced by the detection of additional *I. ventalloi* parasitizing cats in Lisboa and also in Santarém District, where another tick was found infected with *A. phagocytophilum*, as summarized in "PCR-based survey of *Anaplasma phagocytophilum* in Portuguese ticks (Acari: Ixodidae)".

The continued study of Portuguese ticks suggests that *A. phagocytophilum* is not widely distributed but is concentrated in certain environments that either sustain it or possibly allow its continuous introduction. As noted by several authors, wild birds could play a role in the introduction and/or dispersion of *A. phagocytophilum* infected ticks to widespread regions (Alekseev *et al.*, 2001a; Bjoersdorff *et al.*, 2001; Daniels *et al.*, 2002; De la Fuente *et al.*, 2005a). Investigation of this aspect was started during this study, but only a limited number of ticks have been analysed so far. Ticks were collected from wounded birds, recovered in two rehabilitation centers in Lisboa and Setúbal District (mainland), and all specimens were found to be negative for *A. phagocytophilum* in spite of the detection of other tick-borne bacteria, as stated in "Ticks Parasitizing Wild Birds in Portugal: Detection of *Rickettsia aeschlimannii*, *R. helvetica* and *R. massiliae*". Nevertheless, a collaborative agreement between *CEVDI/INSA* and *Instituto de Conservação da Natureza e da Biodiversidade* (*ICNB*) will provide new opportunities for continuing evaluations of the potential for resident, occasional migrant, or migratory birds to maintain ticks and tick-borne agents in Portugal.

During the four years of this study, ticks were obtained from several sampling areas within seven mainland districts, Bragança, Braga, Leiria, Lisboa, Portalegre, Santarém and Setúbal, and also from Madeira Island. The majority of ticks were obtained in collections directed toward areas where the existence of *Ixodes* species was previous documented, as in the Lisboa, Leiria and Setúbal Districts, and on Madeira Island. Additional specimens were obtained as part of ongoing projects with other specific interests, such as from sampling areas in Bragança District. This region was included in a *CEVDI/INSA* project because it had the highest incidence of MSF reported during 1989-2003, yet little was known about the *Rickettsia* species that circulated in this region of the country or about their prevalence in ticks. In Portugal, MSF is the most important tick-borne disease with an incidence rate of 8.9 per $10^5$ inhabitants over 1999-2003 (De Sousa *et al*., 2003). The disease is caused by bacteria belonging to the complex *Rickettsia conorii* which are transmitted by the brown-dog tick, *R. sanguineus* (Bacellar, 1996; Bacellar *et al.*, 1999a, 1999b; De Sousa *et al.*, in press). This tick is widely distributed on the mainland, and according to Baptista's prediction maps, *Rhipicephalus* spp. are especially prevalent in the South, in the Centre and also in Northern inner regions of the country coinciding with the majority of districts reporting the highest prevalences of MSF (De Sousa *et al.*, 2003; Baptista, 2006). By contrast, *I. ricinus* has a more restricted distribution occurring in the littoral region increasing from South to the North (Baptista, 2006). In fact, the samples collected in Bragança District were in accordance with Baptista's data,

showing a low prevalence of *Ixodes* species; although several rickettsiae were found in the studied arthropods, no *A. phagocytophilum* infection was detected. These preliminary results are presented in "Ticks and tick-borne rickettsiae surveillance in Montesinho Natural Park, Portugal" and the results from an additional sample of ticks that was further tested for *A. phagocytophilum* are included in the article that summarized all the tick study work: "PCR-based survey of *Anaplasma phagocytophilum* in Portuguese ticks (Acari: Ixodidae)".

Additionally, the study of *Portalegre* District reinforced the observation of a limited number of *Ixodes* spp. among inland regions of the country when compared to littoral areas. The field work in Portalegre were performed in a similar manner as those in Lisboa and Setúbal districts, i.e. focused in the spring season and carried out over the same number of days, but in those littoral districts *Ixodes* species were found to be generally more abundant. Regardless, due to the exploratory rather than exhaustive nature of this work, it cannot exclude the occurrence of natural foci of *A. phagocytophilum* cycles among inland regions. Although the Continental and Mediterranean climates that generally characterize the North and Southern inland regions are regarded as limiting factors for maintenance of *Ixodes* spp. populations, when compared to the littoral dominated by an oceanic influence, local favourable environmental conditions have been identified. In fact, several authors documented the presence of *I. ricinus*, the main European vector of *A. phagocytophilum*, in all inland districts of Portugal (Dias, 1994; Caeiro, 1999). Interestingly, also in littoral areas *Ixodes* spp. do not seem to present a regular distribution, as in the example of the District of Leiria where only a limited number of specimens were obtained. Again, this patchy distribution may relate to the strict ecological requirements that characterize *Ixodes* spp., so it is possible that some microenvironments that sustain these ticks are not identified when performing sample collections even in areas where these ticks are expected to occur. Madeira Island and *Tapada Nacional de Mafra* (*TNM*), a confined Natural Park belonging to Lisboa District, remain as the few exceptions of this, presenting an abundant tick fauna dominated by *I. ricinus*.

The importance of adequate ecological conditions for the maintenance of *A. phagocytophilum* enzootic cycles, starting with the existence of competent vectors of the genus *Ixodes*, has been demonstrated in all Northern hemisphere countries where this agent is present. Only sporadic reports document *A. phagocytophilum* in other genera of ticks and mites (Des Vignes *et al.*, 1999; Fernandez-Soto *et al.*, 2001; Goethert & Telford, 2003; Holden *et al.*, 2003; Kim *et al.*, 2003; Sarih *et al.*, 2005; Cao *et al.*, 2006; Skoracki *et al.*, 2006), but thus far no study

has proven their role in the agent's transmission, as reviewed in Chapter I. This ecological requirement is also shown here by the exclusive association of *A. phagocytophilum* with *Ixodes* spp., in spite of screening of several other ticks species, some even collected in the same area as infected *Ixodes* ticks, such as Baixa de Palmela in Setúbal District. This study included *Dermacentor marginatus*, *D. reticulatus*, *Hyalomma lusitanicum*, *Haemaphysalis inermis*, *H. puntacta*, *Rhipicephalus bursa*, *R. pusillus*, and the *R. sanguineus* group, which represents 75% of the non-*Ixodes* species present in Portugal, as described in "Carraças associadas a patologias infecciosas em Portugal" (Apendix 1).

Regardless, competent *Ixodes* ticks are found in many regions beyond the areas of pathogen endemicity, and seasonal fluctuations in *A. phagocytophilum*-endemic foci have also been reported. Discrepancies between the presence of vector ticks and pathogen spatial and temporal distribution are not well understood but could be related to arthropod feeding behaviour and reservoir-host dynamics (Swanson *et al.*, 2006). A patchy distribution of *A. phagocytophilum* was also documented in this study. The best example of this phenomenon is shown in *TNM* in opposition to Madeira Island. As previously mentioned, both areas sustain abundant populations of *I. ricinus*, but no active infections by *A. phagocytophilum* were found in *TNM* in the arthropod sampling in spite of the detection of other Rickettsiales associated with *I. ricinus*, as presented in "Detection of *Rickettsia helvetica* and other Spotted Fever Group Rickettsiae in *Ixodes ricinus* from *Tapada Nacional de Mafra*, Portugal". Of interest is the observation that both *TNM* and Madeira Island sustain a great variety of another *I. ricinus*-borne agent, *B. burgdorferi s.l.* including: *B. afzelii*, *B. burgdorferi s.s.*, *B. garinii*, *B. lusitaniae* and *B. valaisiana* in Madeira Island (Matuschka *et al.*, 1994, 1998; Núncio, 2001; Lopes de Carvalho *et al.*, in submission); *B. afzelii*, *B. garinii*, *B. lusitaniae*, and *B. valaisiana* in *TNM* (Baptista *et al.*, 2004, 2006). The apparent lack of *A. phagocytophilum* transovarial transmission in ticks directs attention to competent vertebrates, especially feeding hosts for larvae and nymphs, as critical in the natural maintenance of *A. phagocytophilum* active cycles. Thus, the patchy distribution of *A. phagocytophilum* seems to be multifactorial and probably depends on the existence of both competent vector ticks and reservoir hosts. However, *TNM* possesses a mammal population with small and large-size animals, including rodents, insectivores, carnivores, lagomorphs, and artiodactyls (wild boars, fallow deer and elks), that are capable of acting as both feeding support for ticks and as potential reservoirs for *A. phagocytophilum*. Thus, this aspect alone apparently does not seem to be a limiting factor for

agent maintenance. If further research confirms that the absence of *A. phagocytophilum* in *TNM* is not the result of seasonal fluctuation or methodologic approaches, other aspects should be considered as determinants of the agent's presence and maintenance.

Another interesting finding was the detection of *A. phagocytophilum* variant genotypes in Portuguese ticks. Analysis of *A. phagocytophilum rrs*, *groESL* and *msp2* (*p44*) partial gene sequences obtained from positive ticks demonstrated nucleotide polymorphisms, especially in *groESL* and *msp2*. *A. phagocytophilum* sequences found in Madeira Island *I. ricinus* suggest a close relationship with North American strains isolated from humans, and also with those detected in Central and Northern Europe. Yet, these were divergent from genotypes found in mainland *I. ventalloi*, which represent new variant genotypes of unknown pathogenicity, as detailed in "PCR-based survey of *Anaplasma phagocytophilum* in Portuguese ticks (Acari: Ixodidae)".

The existence of *A. phagocytophilum* genotypes with distinct geographic origins, reservoir hosts and possibly pathogenicity is a significant matter of interest in the scientific community. Ongoing research suggests that genotypes identified in human disease are identical or closely related to those infecting other non-ruminant animals, such as horses, dogs and possibly cats. It is proposed that these genotypes are maintained in an enzootic cycle that is dependent on ticks and small mammals, such as rodents. Thus, to complement the ecological study of *A. phagocytophilum* and address the existence of variant genotypes potentially involved in human granulocytic anaplasmosis in Portugal, several animal populations that could act as reservoirs (rodents) or susceptible hosts (horses and dogs) were screened for active infections, as presented in Chapter IV. To maximize resources and limit the time-consuming and costly collection of samples, biological specimens were obtained from ongoing *CEVDI/INSA* projects or from other independent studies performed by Institutional partners.

The study of potential reservoirs addressed five of the twelve rodent species that are known to inhabit Portugal (Madureira & Ramalhinho, 1981; McDonald & Barret, 1999), including *Apodemus sylvaticus*, *Mus musculus*, *M. spretus*, *Rattus norvegicus* and *R. rattus*. The majority of these species are regarded as hosts for both *I. ricinus* and *I. ventalloi* (Dias, 1994; MM Santos-Silva, personal communication) and were captured in Arrábida, Mafra and Gerês in mainland, Santana and Seixal in Madeira Island, areas where *Ixodes* ticks are known to be present. The additional inland area of Mértola was also included in this study. Antibodies against *A. phagocytophilum* or a closely related agent were detected in *M. spretus* sera from all mainland

sampling areas where this mouse was captured (Arrábida, Mafra and Mértola). The greatest number of seropositive animals was found during spring but, despite the capture season no other proportion with statistically significant was observed, probably due to the limited number of rodents analysed. Spring is recognized as the season when *M. spretus* are most active and is also regarded as the time of the year when *A. phagocytophilum* infections or reinfections occur in rodents (Stafford *et al.*, 1999; Castro *et al.*, 2001). However, active infections by *A. phagocytophilum* were not detected in seropositive animals, as presented in "Detection of antibodies against *Anaplasma phagocytophilum* in wild-rodents, Portugal".

Whether the *M. spretus* seropositivity reflects residual antibodies from past *A. phagocytophilum* infections or active infections with bacteremia lower than detection thresholds deserves a more detailed investigation. The occurrence in ticks of *A. phagocytophilum* ruminant-like genotypes that are regarded to have limited infective potential for rodents was suggested by Massung and coworkers (2003b), and could explain the inability to detect active infection in *M. spretus*. In fact, the dominant ruminant strain (Ap-variant 1) was identified in *I. ricinus* collected from cattle in Spain, as demonstrated in the collaborative study "Detection of a non-pathogenic variant of *Anaplasma phagocytophilum* in *Ixodes ricinus* from La Rioja, Spain". Although ruminant populations were not addressed in this thesis and all genotypes detected in ticks had sequence polymorphisms distinct from the Ap-variant 1 and other ruminant variants, the existence of genotypes with limited infective potential for rodents and other non-ruminants can not be excluded. In this regard, it is of interest the spatial proximity between the area defined as Arrábida in this rodent study, and Baixa de Palmela, where *I. ventalloi* where found infected with a new *A. phagocytophilum* genotype for which pathogenicity potential is unknown. Moreover, in Mafra no *A. phagocytophilum*-infected *Ixodes* spp. could be identified despite the presence of seropositive mice. Thus, the possibility of cross-reactions with antigenically similar agents should also be considered as another explanation for the *M. spretus* serologic results. As further discussed in this chapter, *A. platys,* that is closely related to *A. phagocytophilum*, was recently identified in a Portuguese dog population that had *A. phagocytophilum* serologic reactions. Although the enzootic cycle of this agent remains largely unknown, active infections in rodents are documented in other countries (Chae *et al.*, 2003; Kim *et al.*, 2006).

An important unexplained aspect is the lack of *A. phagocytophilum* detection in Madeira Island rodents despite the documented persistence of this agent in *I. ricinus* ticks in that location. The possibility of inadequate rodent sampling exists due to i) the limited number analysed

compared to the total number of captures, ii) the limited collection studies restricted to only two locations on the Island, or iii) the potential that other unexamined small or medium size animals serve as feeding support for *Ixodes* spp. In fact, both *I. ricinus* and *I. ventalloi* have been detected parasitizing several other feral animals beside rodents, including birds, and members of the Order Carnivora, Erinaceomorpha, Lagomorpha and Soricomorpha (Dias, 1994; MM Santos-Silva, personal communication). Such uncertainties argue for further investigation of *A. phagocytophilum* cycles on Madeira Island.

With regard to possible infections in domestic animals, equine samples were obtained during routine evaluations for serosurveillance of 10 mainland districts, and, seropositive animals were identified in 6, including Braga, Faro, Lisboa, Portalegre, Santarém and Setúbal. Even though no significant association was found between seroprevalence rates and horse origin, age groups or gender, an active *A. phagocytophilum* infection was detected by molecular analysis in one seropositive mare from Faro District, Algarve region, Southern mainland. Nucleotide sequences obtained from this animal exhibited a high degree of similarity to strains involved in human disease, as presented in "Serological survey and molecular detection of *Anaplasma phagocytophilum* infection in Portuguese horses". Additionally, a population of dogs from Algarve region was also tested for active *A. phagocytophilum* infections. These animals were presented to veterinarian with clinical signs compatible of tick-borne disease; the great majority were seroreactive for *A. phagocytophilum*. In five animals the serological criteria for *A. phagocytophilum* active infection were fulfilled by either seroconversion or four-fold increase in antibody titers. Regardless, molecular analysis failed to detect any active *A. phagocytophilum* infections but *A. platys* DNA was found in two seropositive (one presenting seroconversion for *A. phagocytophilum* antigen) and three seronegative animals, as presented in "Anaplasmataceae as etiologic agents of canine tick-borne disease in Portugal".

Horses and dogs are two of the non-ruminant animals that have been documented with granulocytic anaplasmosis in both North American and European areas where *A. phagocytophilum* is known to exist (Engvall *et al.*, 1996; Greig *et al.*, 1996; Madigan *et al.*, 1996; Pusterla *et al.*, 1998a; Bullok *et al.*, 2000; Tozon *et al.*, 2003; Von Loewenich *et al.*, 2003b; Alberti *et al.*, 2005; Lester *et al.*, 2005; Shaw *et al.*, 2005). Identification of infections in these animals is also regarded as a surrogate predictor of human disease. This study provided the first definitive evidence of *A. phagocytophilum* infections in Portuguese horses and suggests the potential occurrence of HGA in

Portugal as well. Despite the lack of active infections in dogs, the close contact of these animals with humans argues for continued evaluations. In a study of patients with dermatological lesions resulting from infection by *Ixodes*-borne agent *B. burgdorferi s.l.*, 1/3 of the individuals owned companion animals, especially dogs but also cats, birds and rodents (Franca, 2004). Moreover, these data add *A. platys* to the list of Anaplasmataceae that circulate in Portugal and emphasize the importance of veterinarians considering not only equine granulocytic anaplasmosis but also canine infectious cyclic thrombocytopenia in the differential diagnosis of tick-borne diseases in Portugal.

Molecular analyses demonstrate that *A. phagocytophilum* with genotypes closely related to those known to cause human disease were found only in *I. ricinus* from Madeira Island and in a horse from the Algarve region. These two geographic regions are well known as tourist destinations and also as nidification or resting sites for several migratory bird species, suggesting the possibility of tick-borne agent introduction, a concept that deserves much more study.

The potential occurrence of human Anaplasmataceae infections in Portugal was first proposed by David de Morais and coworkers (1991). These authors described a febrile patient with negative serological results for typhoid fever, brucellosis, MSF and LB, stable titers for *C. burnetii*, but a >four-fold decrease in *E. chaffeensis* antibodies in three consecutive samples. This finding was reported in the same year that *E. chaffeensis* was first isolated in the US and prior to the published description of the first cases of human granulocytic anaplasmosis; thus, evaluation for *A. phagocytophilum* infection was not performed. The diagnosis was made possible as a consequence of pioneering research initiated at *CEVDI/INSA* by Armindo Filipe, the coordinator of a study of emerging tick-borne agents and their potential impact on human diseases. The determination and enthusiasm of these investigators were undoubtedly the stimuli for development of Anaplasmataceae and other emerging tick-borne disease research in Portugal. In fact, two years before the report of this case that fulfilled serological criteria for *E. chaffeensis* infection, the same investigators documented the first clinical case of LB in Portugal (David de Morais, 1989).

Since the report of the first Portuguese *E. chaffeensis* case, *CEVDI/INSA* has included *E. chaffeensis* IFA as a routine laboratory test available for the community, and over the last year, this was also extended to include testing for *A. phagocytophilum*. Moreover, within the scope of this thesis, the technological exchange with The Johns Hoopkins University School of Medicine.

permitted the implementation of direct tests, including molecular and culture techniques that were performed on an experimentally basis in 2006 and are now being used routinely. In order to alert the practicing medical community about the emergence of Anaplasmataceae, two review articles were published regarding this subject. The first focused on the epidemiological, clinical manifestations, diagnosis and treatment of HME (David de Morais, 1992a). More recently, in response to the increasing detection of *A. phagocytophilum* infections in Europe, an update "Ehrlichiose granulocítica humana. Conceitos actuais" (Apendix 2) was published. To better address whether *A. phagocytophilum* is involved in human infection in Portugal, Chapter V of this thesis is devoted to analysis of biological samples received annually at *CEDVI/INSA* from patients with suspected tick-borne disease.

The occurrence of *A. phagocytophilum* and HGA positively correlates with the geographic distribution of other *Ixodes*-borne agents and associated diseases, such as *B. burgdorferi s.l.*, *Babesia microti* and *B. divergens*, and TBE virus. This correlation is demonstrated by coinfections in ticks (Swanson *et al.*, 2006), the geographic convergence of diseases (CDC, 2001-2006; Dumler, 2005), and increasing recognition of human coinfections (Nadelman *et al.*, 1997; Horowitz *et al.*, 1998a; Krause *et al.*, 2003; Moss & Dumler, 2003; Hermanowska-Szpakowicz *et al.*, 2004; Lotric-Furlan *et al.*, 2005, 2006; Grzeszczuk *et al.*, 2006b). Thus, the first study addresses *A. phagocytophilum* infections in Portuguese patients and is based on a retrospective serological evaluation of samples received in 2002 from individuals with confirmed or suspected LB. Given the non-specific presentation of HGA that is in common with other-tick borne illnesses, an additional cohort representing patients with clinical suspicion of LB/MSF, but lacking laboratory confirmation for these diagnoses was also included. Indeed, this study demonstrated a significantly higher proportion of *A. phagocytophilum* seropositive patients among those with suspected/confirmed LB when compared to the overall population and the control cohort, defined by healthy blood-donors. Although no statistically significant differences in age, gender, or province of origin were noted between *A. phagocytophilum* –seropositive and –seronegative individuals, the majority of samples came from Estremadura region, and among these positive results were more often found in patients who resided in Oeiras than in other locations. Regardless, only a 63 year-old female from Elvas, Alto Alentejo region, who presented with fever and had a >four-fold increase in *A. phagocytophilum* IFA titer in two consecutive samples collected 68 days apart, fulfilled the HGA laboratory diagnostic criteria for active infection (Walker *et al*., 2000; Brouqui *et al.*, 2004). These

results are presented in the article "Human exposure to *Anaplasma phagocytophilum* in Portugal".

This study corroborated previous reports that document a higher *A. phagocytophilum* seroprevalence in at-risk populations, in this case, documented LB patients (reviewed in detail in Chapter I, State of the art). Regardless, for the majority of seropositive patients, *A. phagocytophilum* diagnostic criteria could not be fulfilled because of the lack of paired serum samples. Moreover, application of direct laboratory tests that could definitively confirm HGA, such as isolation and molecular analysis, were not applicable due to the retrospective character of the study and the inavailability of appropriate samples when *A. phagocytophilum* testing was eventually available. The association of seropositivity with Oeiras residence in patients from the Estremadura region is worthy of further consideration. Interestingly, the first human isolate of *B. burgdorferi s.l.* in Portugal, identified as *B. lusitaniae*, was obtained from a patient residing in Oeiras (Collares-Pereira *et al*., 2004; Franca, 2004). Oeiras is located near the urban perimeter of Lisboa city and is one of the administrative divisions of Lisboa District, a littoral area where *Ixodes* spp. are known to occur. This district is also described in several studies as having the highest number of laboratory-confirmed cases of LB in Portugal (Franca, 2004; Baptista, 2006; Lopes de Carvalho & Núncio, 2006), and Oeiras is mentioned in Franca's work (2004) as the residence place of at least three LB patients. The authors discussed potential biases for these results, focusing on the possibility that some patients could acquire infection in districts or countries other than their area of residence while working or in leisure activities (Franca, 2004), or biased by the more ready access to samples from patients of South Central and Southern regions due to the locations of health institute partners and research units where these studies were conducted (Baptista, 2006; Lopes de Carvalho & Núncio, 2006). Nevertheless, the possibility of Lisboa District to be an endemic area for *Ixodes*-borne disease, requires further evaluation.

An additional study analysed available data concerning Anaplasmataceae diagnoses performed in *CEVDI/*INSA during 2000-2006, as presented in "Six years of Anaplasmataceae serodiagnosis in humans. Data from a state laboratory, Portugal". IFA tests for *E. chaffeensis* were performed prospectively as part of routine laboratory diagnosis. Potential cross-reactions between *E. chaffeensis* and *A. phagocytophilum*, as previous documented in both HME and HGA case series (Bakken et al, 1996c; Wong et al, 1997; Comer et al, 1999a; Walls et al, 1999; Lotric-Furlan et al, 2006), prompted testing of samples received based on clinical suspicion of infection

for both agents. Thus, archived sera received during 2000-2005 for *E. chaffeensis* serodiagnosis were analysed retrospectively for the presence of antibodies against *A. phagocytophilum*. In 2006, serological testing was performed prospectively and in parallel for both agents. Such testing demonstrated a higher proportion of *A. phagocytophilum* than *E. chaffeensis* seropositive samples, although reactivity to both agents was also found. These results are in accordance with seroprevalence studies on European population, which suggest greater exposure to *A. phagocytophilum* or an antigenically related agent (Dumler et al, 1997; Groen et al, 2002). For the majority of seropositive patients that had paired sera, stable antibody titers or less than four-fold titer changes were observed. Only one male patient from Setúbal District seroconverted for *A. phagocytophilum*, fulfilling serological criteria for a confirmed case. However neither *A. phagocytophilum* nor *E. chaffeensis* active infections could be detected in the samples analysed by direct techniques.

This study also analysed the results among *A. phagocytophilum* and/or *E. chaffeensis* seropositive patients of tests requested by physicians for other tick-borne agents or related bacteria that were conducted at *CEVDI/INSA*. These supplemental laboratory tests provided serological evidence of bartonellosis in one and acute Q fever cases in five *A. phagocytophilum*-seropositive individuals, rickettsiosis in two *E. chaffeensis*-seropositive patients, and individual cases of acute and chronic Q fever in two patients with both *A. phagocytophilum* and *E. chaffeensis* antibodies. Moreover, culture attempts using samples from a patient with stable *A. phagocytophilum* titers and a *C. burnetii* seroconversion resulted in the isolation of *C. burnetii* in HL-60 cells. Of interest was the observation of another patient with *C. burnetii* seroconversion that also fulfilled serologic criteria for the diagnosis of active *A. phagocytophilum* infection. Whether reactivity to multiple vector-borne agents represents cross-reactivity to shared antigens, such as heat shock proteins or antibodies induced by active coinfection or past exposures to one or more Anaplasmataceae, *C. burnetii*, *Rickettsia* spp., or *Bartonella* spp., remains to be ascertained.

Fifteen years have past since the findings of David de Morais and coworkers (1991), but the question still remains about the interpretation of serologic reactivity to Anaplasmataceae observed among Portuguese patients. For at least *A. phagocytophilum*, potential exists for human infection given identification of genotypes that are similar or closely related to those associated to HGA. However, the possible presence of *E. chaffeensis* or other *Ehrlichia* spp. of medical interest can not be excluded, since this aspect was not investigated. Continued integrated analysis of the results of laboratory diagnostic tests for Anaplasmataceae and other tick-borne infections, with a

special emphasis on applications of direct approaches is indispensable to better understand whether active infections with these agents occur in Portugal. Moreover, the potential for imported cases associated with international travellers to European- or US-endemic areas, underscores the need for continuing active surveillance and vigilance.

# REFERENCES

# REFERENCES


1. ADACHI JA, GRIMM EM, JOHNSON P, UTHMAN M, KAPLAN B, RAKITA RM. Human granulocytic ehrlichiosis in a renal transplant patient: case report and review of the literature. Transplantation. 1997;64(8):1139-42.
2. ADELSON ME, RAO RV, TILTON RC, CABETS K, ESKOW E, FEIN L, OCCI JL, MORDECHAI E. Prevalence of *Borrelia burgdorferi*, *Bartonella* spp., *Babesia microti*, and *Anaplasma phagocytophila* in *Ixodes scapularis* ticks collected in Northern New Jersey. J Clin Microbiol. 2004;42(6):2799-801.
3. AGUERO-ROSENFELD ME. Diagnosis of human granulocytic ehrlichiosis: State of the art. Vector Borne and Zoonotic Dis. 2002;2(4):233-9.
4. AGUERO-ROSENFELD ME, DONNARUMMA L, ZENTMAIER L, JACOB J, FREY M, NOTO R, CARBONARO CA, WORMSER GP. Seroprevalence of antibodies that react with *Anaplasma phagocytophila*, the agent of human granulocytic ehrlichiosis, in different populations in Westchester County, New York. J Clin Microbiol. 2002;40(7):2612-5.
5. AGUERO-ROSENFELD ME, HOROWITZ HW, WORMSER GP, MCKENNA DF, NOWAKOWSKI J, MUNOZ J, DUMLER JS. Human granulocytic ehrlichiosis: a case series from a medical center in New York State. Ann Intern Med. 1996;125(11):904-8.
6. AGUERO-ROSENFELD ME, KALANTARPOUR F, BALUCH M, HOROWITZ HW, MCKENNA DF, RAFFALLI JT, HSIEH T, WU J, DUMLER JS, WORMSER GP. Serology of culture-confirmed case of human granulocytic ehrlichiosis. J Clin Microbiol. 2000;38(2):635-8.
7. AKKOYUNLU M, FIKRIG E. Gamma interferon dominates the murine cytokine response to the agent of human granulocytic ehrlichiosis and helps to control the degree of early rickettsemia. Infect Immun. 2000;68(4):1827-33.
8. AKKOYUNLU M, MALAWISTA SE, ANGUITA J, FIKRIG E. Exploitation of interleukin-8-induced neutrophil chemotaxis by the agent of human granulocytic ehrlichiosis. Infect Immun 2001;69(9):5577-88.
9. ALBERDI MP, WALKER AR, PAXTON EA, SUMPTION KJ. Natural prevalence of infection with *Ehrlichia* (*Cytoecetes*) *phagocytophila* of *Ixodes ricinus* ticks in Scotland. Vet Parasitol. 1998;78(3):203-13.
10. ALBERDI MP, WALKER AR, URQUHART KA. Field evidence that roe deer (*Capreolus capreolus*) are a natural host for *Ehrlichia phagocytophila*. Epidemiol Infect. 2000;124(2):315-23.
11. ALBERTI A, ZOBBA R, CHESSA B, ADDIS MF, SPARAGANO O, PINNA PARPAGLIA ML, CUBEDDU T, PINTORI G, PITTAU M. Equine and canine *Anaplasma phagocytophilum* strains isolated on the island of Sardinia (Italy) are phylogenetically related to pathogenic strains from the United States. Appl Environ Microbiol. 2005;71(10):6418-22.
12. ALEKSEEV AN, DUBININA HV, SEMENOV AV, BOLSHAKOV CV. Evidence of ehrlichiosis agents found in ticks (Acari: Ixodidae) collected from migratory birds. J Med Entomol. 2001b; 38(4):471-4.
13. ALEKSEEV AN, DUBININA HV, VAN DE POL I, SCHOULS LM. Identification of *Ehrlichia* spp. and *Borrelia burgdorferi* in *Ixodes* ticks in the Baltic regions of Russia. J Clin Microbiol. 2001a;39(6):2237-42.
14. ALEXANDRE N. [Estudo clínico e epidemiológico da febre botonosa, ehrlichiose canina e borreliose de Lyme numa população de canídeos domésticos do Algarve]. Tese de Mestrado em Saúde Pública Veterinária, Faculdade de Medicina Veterinária, Universidade Técnica de Lisboa, Lisboa, 2006. [MSc Thesis in Portuguese and abstract in English].
15. ALMEIDA, V. [Ixodídeos da zona planaltica do Paul da Serra. Contribuição ao estudo da sua ecologia]. Méd Vet 1996;46:5-22. [Article in Portuguese]
16. ANDERSON BE, DAWSON JE, JONES DC, WILSON KH. *Ehrlichia chaffeensis*, a new species associated with human ehrlichiosis. J Clin Microbiol. 1991;29(12):2838-42.
17. ANDERSON BE, GREENE CE, JONES DC, DAWSON JE. *Ehrlichia ewingii* sp. nov., the etiologic agent of canine granulocytic ehrlichiosis. Int J Syst Bacteriol. 1992;42(2):299-302.
18. ARNEZ M, PETROVEC M, LOTRIC-FURLAN S, ZUPANC TA, STRLE F. First European pediatric case of human granulocytic ehrlichiosis. J Clin Microbiol. 2001;39(12):4591-2.
19. BACELLAR, FC. [Rickettsias isoladas em Portugal. Contribuição para a identificação e classificação de estirpes]. Tese de Doutoramento, Universidade de Évora, Évora, 1996. [PhD Thesis in Portuguese and abstract in English].
20. BACELLAR FC. Ticks and Spotted Fever Rickettsiae in Portugal. *In*: Rickettsiae and Rickettsial Diseases at the Turn of the Third Millenium. Raoult D, Brouqui P, editors. Paris: Elsevier; 1999a. p. 423-7.

21. BACELLAR FC, BEATI L, FRANÇA A, POÇAS J, REGNERY R, FILIPE AR: Israeli spotted fever Rickettsia (*Rickettsia conorii* complex) associated with humam diseses in Portugal. Emerg Infect Dis. 1999b; 5(6):835-6.
22. BACELLAR F, DAWSON JE, SILVEIRA CA, FILIPE AR. Antibodies against Rickettsiaceae in dogs of Setúbal, Portugal. Cent Eur J Public Health. 1995;3(2):100-2.
23. BAKKEN JS, DUMLER JS. Human granulocytic ehrlichiosis. Clin Infect Dis. 2000;31(2):554-60.
24. BAKKEN JS, DUMLER JS. Clinical diagnosis and treatment of human granulocytotropic anaplasmosis. Ann NY Acad Sci. 2006;1078(1):236-47.
25. BAKKEN JS, AGUERO-ROSENFELD ME, TILDEN RL, WORMSER GP, HOROWITZ HW, RAFFALLI JT, BALUCH M, RIDDELL D, WALLS JJ, DUMLER JS. Serial measurements of hematologic counts during the active phase of human granulocytic ehrlichiosis. Clin Infect Dis. 2001;32(6):862-70.
26. BAKKEN JS, DUMLER JS, CHEN SM, ECKMAN MR, VAN ETTA LL, WALKER DH. Human granulocytic ehrlichiosis in the upper Midwest United States: a new species emerging? JAMA. 1994;272(3):212–8.
27. BAKKEN JS, ERLEMEYER SA, KANOFF RJ, SILVESTRINI TC 2ND, GOODWIN DD, DUMLER JS. Demyelinating polyneuropathy associated with human granulocytic ehrlichiosis. Clin Infect Dis. 1998a;27(5):1323-4.
28. BAKKEN JS, GOELLNER P, VAN ETTEN M, BOYLE DZ, SWONGER OL, MATTSON S, KRUETH J, TILDEN RL, ASANOVICH K, WALLS J, DUMLER JS. Seroprevalence of human granulocytic ehrlichiosis among permanent residents of northwestern Wisconsin. Clin Infect Dis. 1998b;27(6):1491-6.
29. BAKKEN JS, HALLER I, RIDDELL D, WALLS JJ, DUMLER JS. The serological response of patients infected with the agent of human granulocytic ehrlichiosis. Clin Infect Dis. 2002;34(1):22-7.
30. BAKKEN JS, KRUETH J, LUND T, MALKOVITCH D, ASANOVICH K, DUMLER JS. Exposure to deer blood may be a cause of human granulocytic ehrlichiosis. Clin Infect Dis. 1996a;23(1):198.
31. BAKKEN JS, KRUETH J, TILDEN JL, DUMLER JS, KRISTIANSEN BE. Serological evidence of human granulocytic ehrlichiosis in Norway. Eur J Clin Microbiol Infect Dis. 1996b;15(10): 829-32.
32. BAKKEN JS, KRUETH J, WILSON-NORDSKOG C, TILDEN RL, ASANOVICH K, DUMLER JS. Clinical and laboratory characteristics of human granulocytic ehrlichiosis. JAMA. 1996c;275(3):199–205.
33. BANERJEE R, ANGUITA J, ROOS D, FIKRIG E. Cutting edge: infection by the agent of human granulocytic ehrlichiosis prevents the respiratory burst by down-regulating gp91phox. J Immunol. 2000;164(8):3946-9.
34. BAPTISTA S. [Lyme borreliosis in Portugal. Study on vector(s), agent(s) and risk factors]. Tese de Doutoramento, Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa, Lisboa, 2006. [PhD Thesis in English].
35. BAPTISTA S, QUARESMA A, AIRES T, KURTENBACH K, SANTOS-REIS M, NICHOLSON M, COLLARES-PEREIRA M. Lyme borreliosis spirochetes in questing ticks from mainland Portugal. Int J Med Microbiol. 2004;293(37):109-16.
36. BARLOUGH JE, MADIGAN JE, DEROCK E, DUMLER JS, BAKKEN JS. Protection against *Ehrlichia equi* is conferred by prior infection with the human granulocytic ehrlichia (HGE agent). J Clin Microbiol. 1995;33(12):3333–4.
37. BARLOUGH JE, MADIGAN JE, KRAMER VL, CLOVER JR, HUI LT, WEBB JP, VREDEVOE LK. *Ehrlichia phagocytophila* genogroup rickettsiae in ixodid ticks from California collected in 1995 and 1996. J Clin Microbiol. 1997a;35(8):2018-21.
38. BARLOUGH JE, MADIGAN JE, TUROFF DR, CLOVER JR, SHELLY SM, DUMLER JS. An *Ehrlichia* strain from a llama (*Lama glama*) and Llama-associated ticks (*Ixodes pacificus*). J Clin Microbiol. 1997b;35(4):1005-7.
39. BAUMGARTEN BU, ROLLINGHOFF M, BOGDAN C. Prevalence of *Borrelia burgdorferi* and granulocytic and monocytic ehrlichiae in *Ixodes ricinus* ticks from southern Germany. J Clin Microbiol. 1999;37(11):3448-51.
40. BAYARD-MC NEELEY M, BANSAL A, CHOWDHURY I, GIRAO G, SMALL CB, SEITER K, NELSON J, LIVERIS D, SCHWARTZ I, MC NEELEY DF, WORMSER GP, AGUERO-ROSENFELD ME. *In vivo* and *in vitro* studies on *Anaplasma phagocytophilum* infection of the myeloid cells of a patient with chronic myelogenous leukaemia and human granulocytic ehrlichiosis. J Clin Pathol. 2004;57(5):499-503.
41. BELONGIA EA. Epidemiology and impact of coinfections acquired from *Ixodes* ticks. Vector Borne Zoonotic Dis. 2002;2(4):265-73.
42. BELONGIA EA, GALE CM, REED KD, MITCHELL PD, VANDERMAUSE M, FINKEL MF, KAZMIERCZK JJ, DAVIS J. Population-based incidence of human granulocytic ehrlichiosis in northwestern Wisconsin, 1997-1999. J Infect Dis. 2001A 184(11):1470–1474.
43. BELONGIA EA, REED KD, MITCHELL PD, CHYOU PH, MUELLER-RIZNER N, FINKEL MF, SCHRIEFER ME. Clinical and epidemiological features of early Lyme disease and human granulocytic ehrlichiosis in Wisconsin. Clin Infect Dis. 1999;29(6):1472-7.
44. BELONGIA EA, REED KD, MITCHELL PD, KOLBERT CP, PERSING DH, GILL JS, KAZMIERCZAK JJ. Prevalence of granulocytic *Ehrlichia* infection among white-tailed deer in Wisconsin. J Clin Microbiol. 1997;35(6):1465-8.

45. BELONGIA EA, REED KD, MITCHELL PD, MUELLER-RIZNER N, VANDERMAUSE M, FINKEL MF, KAZMIERCZAK JJ. Tick-borne infections as a cause of nonspecific febrile illness in Wisconsin. Clin Infect Dis. 2001b;32(10):1434-9.
46. BELTRAME A, RUSCIO M, ARZESE A, RORATO G, NEGRI C, LONDERO A, CRAPIS M, SCUDELLER L, VIALE P. Human granulocytic anaplasmosis in northeastern Italy. Ann NY Acad Sci. 2006;1078:106-9.
47. BJOERSDORFF A, BAGERT B, MASSUNG RF, GUSA A, ELIASSON I. Isolation and characterization of two European strains of *Ehrlichia phagocytophila* of equine origin. Clin Diagn Lab Immunol. 2002a;9(2):341-3.
48. BJOERSDORFF A, BERGLUND J, KRISTIANSEN BE, SODERSTROM C, ELIASSON I. Varying clinical picture and course of human granulocytic ehrlichiosis. Twelve Scandinavian cases of the new tick-borne zoonosis are presented] Lakartidningen. 1999a;96(39):4200-4. [Article in Swedish and abstract in English].
49. BJOERSDORFF A, BERGSTROM S, MASSUNG RF, HAEMIG PD, OLSEN B. Ehrlichia-infected ticks on migrating birds. Emerg Infect Dis. 2001;7(5):877-9.
50. BJOERSDORFF A, BROUQUI P, ELIASSON I, MASSUNG RF, WITTESJO EB, BERGLUND J. Serological evidence of *Ehrlichia* infection in Swedish Lyme borreliosis patients. Scand J Infect Dis. 1999b;31(1):51- 5.
51. BJOERSDORFF A, SVENDENIUS L, OWENS JH, MASSUNG RF. Feline granulocytic ehrlichiosis--a report of a new clinical entity and characterisation of the infectious agent. J Small Anim Pract. 1999c;40(1):20-4.
52. BJOERSDORFF A, WITTESJO B, BERGLUN J, MASSUNG RF, ELIASSON I. Human granulocytic ehrlichiosis as a common cause of tick-associated fever in Southeast Sweden: report from a prospective clinical study. Scand J Infect Dis. 2002b;34(3):187-91.
53. BLAGBURN BL, SPENCER JA, BILLETER SA, DRAZENOVICH NL, BUTLER JM, LAND TM, DYKSTRA CC, STAFFORD KC, POUGH MB, LEVY SA, BLEDSOE DL. Use of imidacloprid-permethrin to prevent transmission of *Anaplasma phagocytophilum* from naturally infected *Ixodes scapularis* ticks to dogs. Vet Ther. 2004;5(3):212-7.
54. BLANCO JR, OTEO JA. Human granulocytic ehrlichiosis in Europe. Clin Microbiol Infect. 2002;8(12):763-72.
55. BLOUIN EF, KOCAN KM. Morphology and development of *Anaplasma marginale* (Rickettsiales: Anaplasmataceae) in cultured *Ixodes scapularis* (Acari:Ixodidae) cells. J Med Entomol. 1998;35(5):788–97.
56. BOER R, VAN DEN BOGAARD AE. Removal of attached nymphs and adults of *Ixodes ricinus* (Acari: Ixodidae). J Med Entomol. 1993;30(4):748-52.
57. BORJESSON DL, BARTHOLD SW. The mouse as a model for investigation of human granulocytic ehrlichiosis: current knowledge and future directions. Comp Med. 2002;52(5):403-13.
58. BORJESSON DL, KOBAYASHI SD, WHITNEY AR, VOYICH JM, ARGUE CM, DELEO FR. Insights into pathogen immune evasion mechanisms: *Anaplasma phagocytophilum* fails to induce an apoptosis differentiation program in human neutrophils. J Immunol. 2005;174(10):6364-72.
59. BRANGER S, ROLAIN JM, RAOULT D. Evaluation of antibiotic susceptibilities of *Ehrlichia canis*, *Ehrlichia chaffeensis*, and *Anaplasma phagocytophilum* by real-time PCR. Antimicrob Agents Chemother. 2004;48(12):4822-8.
60. BROUQUI P, BACELLAR F, BARANTON G, BIRTLES RJ, BJOERSDORFF A, BLANCO JR, CARUSO G, CINCO M, FOURNIER PE, FRANCAVILLA E, JENSENIUS M, KAZAR J, LAFERL H, LAKOS A, LOTRIC FURLAN S, MAURIN M, OTEO JA, PAROLA P, PEREZ-EID C, PETER O, POSTIC D, RAOULT D, TELLEZ A, TSELENTIS Y, WILSKE B; ESCMID Study Group on *Coxiella*, *Anaplasma*, *Rickettsia* and *Bartonella*; European Network for Surveillance of Tick-Borne Diseases. Guidelines for the diagnosis of tick-borne bacterial diseases in Europe. Clin Microbiol Infect. 2004;10(12):1108-32.
61. BROUQUI P, DUMLER JS, LIENHARD R, BROSSARD M, RAOULT D. Human granulocytic ehrlichiosis in Europe. Lancet. 1995;346(8977):782-3.
62. BROUQUI P, SALVO E, DUMLER JS, RAOULT D. Diagnosis of granulocytic ehrlichiosis in humans by immunofluorescence assay. Clin Diagn Lab Immunol. 2001;8(1):199-202.
63. BROUQUI P, SANOGO YO, CARUSO G, MEROLA F, RAOULT D. Candidatus *Ehrlichia walkerii*: a new *Ehrlichia* detected in *Ixodes ricinus* tick collected from asymptomatic humans in Northern Italy. Ann NY Acad Sci. 2003;990:134-40.
64. BOWN KJ, BEGON M, BENNETT M, BIRTLES RJ, BURTHE S, LAMBIN X, TELFER S, WOLDEHIWET Z, OGDEN NH. Sympatric *Ixodes trianguliceps* and *Ixodes ricinus* ticks feeding on field voles (*Microtus agrestis*): potential for increased risk of *Anaplasma phagocytophilum* in the United Kingdom? Vector Borne Zoonotic Dis. 2006;6(4):404-10.
65. BOWN KJ, BEGON M, BENNETT M, WOLDEHIWET Z, OGDEN NH. Seasonal dynamics of *Anaplasma phagocytophila* in a rodent-tick (*Ixodes trianguliceps*) system, United Kingdom. Emerg Infect Dis. 2003;9(1):63-70.

66. BUITRAGO MI, IJDO JW, RINAUDO P, SIMON H, COPEL J, GADBAW J, HEIMER R, FIKRIG E, BIA FJ. Human granulocytic ehrlichiosis during pregnancy treated successfully with rifampin. Clin Infect Dis. 1998;27(1):213–15.
67. BULLER RS, ARENS M, HMIEL SP, PADDOCK CD, SUMNER JW, RIKHISA Y, UNVER A, GAUDREAULT-KEENER M, MANIAN FA, LIDDELL AM, SCHMULEWITZ N, STORCH GA. *Ehrlichia ewingii*, a newly recognized agent of human ehrlichiosis. N Engl J Med. 1999;341(3):148-55.
68. BULLOCK PM, AMES TR, ROBINSON RA, GREIG B, MELLENCAMP MA, DUMLER JS. *Ehrlichia equi* infection of horses from Minnesota and Wisconsin: detection of seroconversion and acute disease investigation. J Vet Intern Med. 2000;14(3):252-7.
69. BUNNELL JE, TRIGIANI ER, SRINIVAS SR, DUMLER JS. Development and distribution of pathologic lesions are related to immune status and tissue deposition of human granulocytic ehrlichiosis agent-infected cells in a murine model system. J Infect Dis. 1999;180(2):546-50.
70. BURKOT TR, MAUPIN GO, SCHNEIDER BS, DENATALE C, HAPP CM, RUTHERFORD JS, ZEIDNER NS. Use of a sentinel host system to study the questing behavior of *Ixodes spinipalpis* and its role in the transmission of *Borrelia bissettii*, human granulocytic ehrlichiosis, and *Babesia microti*. Am J Trop Med Hyg. 2001;65(4):293-9.
71. CAEIRO V. General review of tick species present in Portugal. Parassitologia. 1999;41(1):11-5.
72. CAMICAS J-L, HERVY J-P, ADAM F, MOREL P-C. In: The ticks of the World: Nomenclature, Described stages, Hosts, Distribution (Acarina, Ixodida). Institut Français de Recherche Scientifique pour Le Développement En Coopération. Paris: Éditions de l'Órstom; 1998. p.1-233.
73. CAO WC, ZHAO QM, ZHANG PH, DUMLER JS, ZHANG XT, FANG LQ, YANG H. Granulocytic ehrlichiae in *Ixodes persulcatus* ticks from an area in China where Lyme disease is endemic. J Clin Microbiol. 2000;38(11):4208–10.
74. CAO WC, ZHAO QM, ZHANG PH, YANG H, WU XM, WEN BH, ZHANG XT, HABBEMA JD. Prevalence of *Anaplasma phagocytophila* and *Borrelia burgdorferi* in *Ixodes persulcatus* ticks from northeastern China. Am J Trop Med Hyg. 2003;68(5):547-50.
75. CAO WC, ZHAN L, HE J, FOLEY JE, DE VLAS SJ, WU XM, YANG H, RICHARDUS JH, HABBEMA JD. Natural *Anaplasma phagocytophilum* infection of ticks and rodents from a forest area of Jilin Province, China. Am J Trop Med Hyg. 2006;75(4):664-8.
76. CAPORALE DA, JOHNSON CM, MILLARD BJ. Presence of *Borrelia burgdorferi* (Spirochaetales: Spirochaetaceae) in southern Kettle Moraine State Forest, Wisconsin, and characterization of strain W97F51. J Med Entomol. 2005;42(3):457-72.
77. CARPANO M. Piroplasmosis in Egyptian fowles (*Egyptianella pullorum*). Vet Serv Bull Egypt Minist Agric Sci Technol Serv. 1928;86:1–7.
78. CASTRO MB, NICHOLSON WL, KRAMER VL, CHILDS JE. Persistent infection in *Neotoma fuscipes* (Muridae: Sigmodontinae) with *Ehrlichia phagocytophila sensu lato*. Am J Trop Med Hyg. 2001;65(4):261-7.
79. CARLYON JA, FIKRIG E. Invasion and survival strategies of *Anaplasma phagocytophilum*. Cell Microbiol. 2003;5(11):743-54.
80. CARLYON JA, FIKRIG E. Mechanisms of evasion of neutrophil killing by *Anaplasma phagocytophilum*. Curr Opin Hematol. 2006;13(1):28-33.
81. CARLYON JA, ABDEL-LATIF D, PYPAERT M, LACY P, FIKRIG E. *Anaplasma phagocytophilum* utilizes multiple host evasion mechanisms to thwart NADPH oxidase-mediated killing during neutrophil infection. Infect Immun. 2004;72(8):4772-83.
82. CARLYON JA, CHAN WT, GALAN J, ROOS D, FIKRIG E. Repression of rac2 mRNA expression by *Anaplasma phagocytophila* is essential to the inhibition of superoxide production and bacterial proliferation. J Immunol 2002;169(12):7009-18.
83. CASPERSEN K, PARK J-H, PATIL S, DUMLER JS. Genetic variability and stability of *Anaplasma phagocytophila msp2* (*p44*). Infect Immun. 2002;70(3):1230-4.
84. CATUREGLI P, ASANOVICH KM, WALLS JJ, BAKKEN JS, MADIGAN JE, POPOV VL, DUMLER JS. ankA: an *Ehrlichia phagocytophila* group gene encoding a cytoplasmic protein antigen with ankyrin repeats. Infect Immun. 2000;68(9):5277-83.
85. CDC-Centers for Disease Control and Prevention. Seizures temporally associated with use of DEET insect repellent: New York and Connecticut. MMWR Morb Mortal Wkly Rep. 1989;38:678–80.
86. CDC-Centers for Disease Control and Prevention. Human granulocytic ehrlichiosis - New York, 1995. Morb Mortal Wkly Rep. 1995;44:593-5.
87. CDC-Centers for Disease Control and Prevention. Statewide surveillance for ehrlichiosis - Connecticut and New York, 1994–1997. Morb Mortal Wkly Rep. 1998;479:476–80.
88. CDC – CENTERS FOR DISEASE CONTROL AND PREVENTION. Summary of notifiable diseases, United States, 1999. Morb Mortal Wkly Rep. 2001;48(53):1-106.
89. CDC – CENTERS FOR DISEASE CONTROL AND PREVENTION. Summary of notifiable diseases, United States, 2000. Morb Mortal Wkly Rep. 2002;49(53):1-104.
90. CDC – CENTERS FOR DISEASE CONTROL AND PREVENTION. Summary of notifiable diseases, United States, 2001. Morb Mortal Wkly Rep. 2003;50(53):1-110.
91. CDC – CENTERS FOR DISEASE CONTROL AND PREVENTION. Summary of notifiable diseases, United States, 2002. Morb Mortal Wkly Rep. 2004;51(53):1-86.

92. CDC – CENTERS FOR DISEASE CONTROL AND PREVENTION. Summary of notifiable diseases, United States, 2003. Morb Mortal Wkly Rep. 2005;52(54):1-86.
93. CDC – CENTERS FOR DISEASE CONTROL AND PREVENTION. Summary of notifiable diseases, United States, 2004. Morb Mortal Wkly Rep. 2006;53(53):1-82.
94. CENTENO-LIMA S, DO ROSARIO V, PARREIRA R, MAIA AJ, FREUDENTHAL AM, NIJHOF AM, JONGEJAN F. A fatal case of human babesiosis in Portugal: molecular and phylogenetic analysis. Trop Med Int Health. 2003;8(8):760-4.
95. CHAE JS, FOLEY JE, DUMLER JS, MADIGAN JE. Comparison of the nucleotide sequences of 16S rRNA, 444 Ep-ank, and *groESL* heat shock operon genes in naturally occurring *Ehrlichia equi* and human granulocytic ehrlichiosis agent isolates from Northern California. J Clin Microbiol. 2000;38(4):1364-9.
96. CHAE JS, KIM CM, KIM EH, HUR EJ, KLEIN TA, KANG TK, LEE HC, SONG JW. Molecular epidemiological study for tick-borne disease (*Ehrlichia* and *Anaplasma* spp.) surveillance at selected US Military Training Sites/Installations in Korea. Ann NY Acad Sci. 2003;990:118-25.
97. CHANG YF, NOVOSEL V, CHANG CF, KIM JB, SHIN SJ, LEIN DH. Detection of human granulocytic ehrlichiosis agent and *Borrelia burgdorferi* in ticks by polymerase chain reaction. J Vet Diagn Invest. 1998;10(1):56-9.
98. CHAPMAN AS, BAKKEN JS, FOLK SM, PADDOCK CD, BLOCH KC, KRUSELL A, SEXTON DJ, BUCKINGHAM SC, MARSHALL GS, STORCH GA, DASCH GA, MCQUISTON JH, SWERDLOW DL, DUMLER SJ, NICHOLSON WL, WALKER DH, EREMEEVA ME, OHL CA; Tickborne Rickettsial Diseases Working Group; CDC. Diagnosis and management of tickborne rickettsial diseases: Rocky Mountain spotted fever, ehrlichioses, and anaplasmosis—United States. A practical guide for physicians and other health-care professionals. MMWR Recomm Rep. 2006;55(RR-4):1–27.
99. CHEN S-M, DUMLER JS, BAKKEN JS, WALKER DH. Identification of a granulocytotropic *Ehrlichia* species as the etiologic agent of human disease. J Clin Microbiol. 1994;32(3):589-95.
100. CHOI KS, DUMLER JS. Early induction and late abrogation of respiratory burst in *A. phagocytophilum*-infected neutrophils. Ann NY Acad Sci. 2003;990:488-93.
101. CHOI KS, GARYU J, PARK J, DUMLER JS. Diminished adhesion of *Anaplasma phagocytophilum*-infected neutrophils to endothelial cells is associated with reduced expression of leukocyte surface selectin. Infect Immun. 2003;71(8):4586-94.
102. CHOI KS, GRAB DJ, DUMLER JS. *Anaplasma phagocytophilum* infection induces protracted neutrophil degranulation. Infect Immun. 2004;72(6):3680-3.
103. CHOI KS, WEBB T, OELKE M, SCORPIO DG, DUMLER JS. Differential innate immune cell activation and proinflammatory response in *Anaplasma phagocytophilum* infection. Infect Immun. 2007;75:3124-30.
104. CHRISTOVA IS, DUMLER SJ. Human granulocytic ehrlichiosis in Bulgaria. Am J Trop Med Hyg. 1999; 60(1):58–61.
105. CHRISTOVA I, GLADNISHKA T. Prevalence of infection with *Francisella tularensis*, *Borrelia burgdorferi sensu lato* and *Anaplasma phagocytophilum* in rodents from an endemic focus of tularemia in Bulgaria. Ann Agric Environ Med. 2005;12(1):149-52.
106. CHRISTOVA I, SCHOULS L, VAN DE POL I, PARK J, PANAYOTOV S, LEFTEROVA V, KANTARDJIEV T, DUMLER JS. High prevalence of granulocytic ehrlichiae and *Borrelia burgdorferi* sensu lato in *Ixodes ricinus* ticks from Bulgaria. J Clin Microbiol. 2001;39(11):4172-4.
107. CINCO M, PADOVAN D, MURGIA R, MAROLI M, FRUSTERI L, HELDTANDER M, JOHANSSON KE, ENGVALL EO. Coexistence of *Ehrlichia phagocytophila* and *Borrelia burgdorferi sensu lato* in *Ixodes ricinus* from Italy as determined by 16S rRNA gene sequencing. J Clin Microbiol. 1997;35(12):3365-6.
108. CISAK E, CHMIELEWSKA-BADORA J, ZWOLINSKI J, WOJCIK-FATLA A, POLAK J, DUTKIEWICZ J. Risk of tick-borne bacterial diseases among workers of Roztocze National Park (south-eastern Poland). Ann Agric Environ Med. 2005;12(1):127-32.
109. CIZMAN M, AVSIC-ZUPANC T, PETROVEC M, RUZIC-SABLJIC E, POKORN M. Seroprevalence of ehrlichiosis, Lyme borreliosis and tick-borne encephalitis infections in children and young adults in Slovenia. Wien Klin Wochenschr. 2000;112(19):842-5.
110. COLLARES-PEREIRA M, COUCEIRO S, FRANCA I, KURTENBACH K, SCHAFER SM, VITORINO L, GONÇALVES L, BAPTISTA S, VIEIRA ML, CUNHA C. First Isolation of *Borrelia lusitaniae* from a human patient. J Clin Microbiol. 2004;42(3):1316-8.
111. COLLARES-PEREIRA M, FRANCA I. [Borreliose de Lyme: Ocorrência em Portugal]. Trab Soc Port Dermatol Venerol. 2000;58(Suppl):107-16. [Article in Portuguese and abstract in English].
112. COMER JA, NICHOLSON WL, OLSON JG, CHILDS JE. Serologic testing for human granulocytic ehrlichiosis at a National Referral Center. J Clin Microbiol. 1999a;37(3):558-64.
113. COMER JA, NICHOLSON WL, SUMNER JW, OLSON JG, CHILDS JE. Diagnosis of human ehrlichiosis by PCR assay of acute-phase serum. J Clin Microbiol. 1999b;37(1):31-4.

114. COURTNEY JW, DRYDEN RL, MONTGOMERY J, SCHNEIDER BS, SMITH G, MASSUNG RF. Molecular characterization of *Anaplasma phagocytophilum* and *Borrelia burgdorferi* in *Ixodes scapularis* ticks from Pennsylvania. J Clin Microbiol. 2003;41(4):1569-73.
115. COWDRY EV. Studies on the aetiology of heartwater. I. Observation of rickettsia, *Rickettsia ruminantium* (n.sp.) in the tissues of infected animals. J Exp Med. 1925;44:803-4.
116. DA COSTA PS, VALLE LM, BRIGATTE ME, GRECO DB. More about human monocytotropic ehrlichiosis in Brazil: serological evidence of nine new cases. Braz J Infect Dis. 2006;10(1):7-10.
117. DAME JB, MAHAN SM, YOWELL CA. Phylogenetic relationship of *Cowdria ruminantium*, agent of heartwater, to *Anaplasma marginale* and other members of the order Rickettsiales determined on the basis of 16S rRNA sequence. Int J Syst Bacteriol. 1992;42: 270-4.
118. DANIELS TJ, BATTALY GR, LIVERIS D, FALCO RC, SCHWARTZ I. Avian reservoirs of the agent of human granulocytic ehrlichiosis? Emerg Infect Dis. 2002;8(12):1524-5.
119. DANIELS TJ, BOCCIA TM, VARDE S, MARCUS J, LE J, BUCHER DJ, FALCO RC, SCHWARTZ I. Geographic risk for lyme disease and human granulocytic ehrlichiosis in southern New York state. Appl Environ Microbiol. 1998;64(12):4663-9.
120. DANIEL SA, MANIKA K, ARVANITIDOU M, DIZA E, SYMEONIDIS N, ANTONIADIS A. Serologic evidence of human granulocytic ehrlichiosis, Greece. Emerg Infect Dis. 2002b;8(6):643-4.
121. DAVID DE MORAIS JA. [Epidemiologia e clínica da ehrlichiose humana. A propósito do primeiro caso diagnosticado na Europa]. Rev Port Doen Infec. 1992a;15(1):7-18. [Article in Portuguese and abstract in English].
122. DAVID DE MORAIS JA, ABRANCHES J, PARRA J, FILIPE AR, NÚNCIO MS, SILVA MH, VALADAS E. [Artrite de Lyme. A propósito dos primeiros casos diagnosticados em Portugal]. Rev Port Doen Infec. 1994;17(3):183-95. [Article in Portuguese and abstract in English].
123. DAVID DE MORAIS JD, DAWSON JE, GREENE C, FILIPE AR, GALHARDAS LC, BACELLAR F. First European cases of ehrlichiosis. Lancet. 1991;338:633–4.
124. DAVID DE MORAIS J, DAWSON JE, GREENE C, FILIPE AR, GALHARDAS LC, BACELLAR F. [Ehrlichiose humana. Primeiro caso diagnosticado na Europa (Portugal)]. Rev Port Doen Infec. 1992b;15 (1):59-63. [Article in Portuguese and abstract in English].
125. DAVID DE MORAIS JA, FILIPE AR, NÚNCO MS. [Doença de Lyme em Portugal. Caso clínico]. Rev Port Doen Infec. 1989;12:261-76. [Article in Portuguese and abstract in English].
126. DAVID DE MORAIS JA, LEITÃO A, PÁSCOA B, FILIPE AR, NÚNCIO MS. [Doença de Lyme]. Rev Port Doen Infec. 1992c;15 (4):227-45. [Article in Portuguese and abstract in English].
127. DAWSON JE, ANDERSON BE, FISHBEIN DB, SANCHEZ JL, GOLDSMITH CS, WILSON KH, DUNTLEY CW. Isolation and characterization of an *Ehrlichia* sp. from a patient diagnosed with human ehrlichiosis. J Clin Microbiol. 1991;29(12):2741-5.
128. DAWSON JE, BIGGIE KL, WARNER CK, COOKSON K, JENKINS S, LEVINE JF, OLSON JG. Polymerase chain reaction evidence of *Ehrlichia chaffeensis*, an etiologic agent of human ehrlichiosis, in dogs from Southeast Virginia. Am J Vet Res. 1996;57(8):1175-9.
129. DE LA FUENTE J, NARANJO V, RUIZ-FONS F, HOFLE U, FERNANDEZ DE MERA IG, VILLANUA D, ALMAZAN C, TORINA A, CARACAPPA S, KOCAN KM, GORTAZAR C. Potential vertebrate reservoir hosts and invertebrate vectors of *Anaplasma marginale* and *A. phagocytophilum* in central Spain. Vector Borne Zoonotic Dis. 2005a;5(4):390-401.
130. DE LA FUENTE J, TORINA A, NARANJO V, CARACAPPA S, DI MARCO V, ALONGI A, RUSSO M, MAGGIO AR, KOCAN KM. Infection with *Anaplasma phagocytophilum* in a seronegative patient in Sicily, Italy: case report. Ann Clin Microbiol Antimicrob. 2005b;4:15.
131. DE SOUSA R, NÓBREGA SD, BACELLAR F, TORGAL J. [Sobre a realidade epidemiologica da febre escaro-nodular em Portugal]. Acta Medica. 2003;16:430-8. [Article in Portuguese and abstract in English].
132. DE SOUSA R, SANTOS-SILVA M, SANTOS AS, TORGAL J, WALKER DH, BACELLAR F. Israeli tick typhus strain isolated from *Rhipicephalus sanguineus* ticks in Portugal. Vector-Borne and Zoonotic Dis. In press.
133. DEMMA LJ, HOLMAN RC, MCQUISTON JH, KREBS JW, SWERDLOW DL. Epidemiology of human ehrlichiosis and anaplasmosis in the United States, 2001-2002. Am J Trop Med Hyg. 2005;73(2):400-9.
134. DE MICHELIS S, SEWEL HS, COLARES-PEREIRA M, SANTOS-REIS M, SHOULS LM, BENES V, HOLMES EC, KURTENBACH K. Genetic diversity of *Borrelia burgdorferi sensu lato* in ticks from mainland Portugal. J Clin Microbiol. 2000;38:2128-33.
135. DENATALE CE, BURKOT TR, SCHNEIDER BS, ZEIDNER NS. Novel potential reservoirs for *Borrelia* sp. and the agent of human granulocytic ehrlichiosis in Colorado. J Wildl Dis. 2002;38(2):478-82.
136. DERDAKOVA M, HALANOVA M, STANKO M, STEFANCIKOVA A, CISLAKOVA L, PET'KO B. Molecular evidence for *Anaplasma phagocytophilum* and *Borrelia burgdorferi* sensu lato in *Ixodes ricinus* ticks from eastern Slovakia. Ann Agric Environ Med. 2003;10(2):269-71.
137. DES VIGNES F, FISH D. Transmission of the agent of human granulocytic ehrlichiosis by host-seeking *Ixodes scapularis* (Acari: Ixodidae) in Southern New York State. J Med Entomol. 1997;34(4):379-82.

138. DES VIGNES F, LEVIN ML, FISH D. Comparative vector competence of *Dermacentor variabilis* and *Ixodes scapularis* (Acari: Ixodidae) for the agent of human granulocytic ehrlichiosis. J Med Entomol. 1999;36:182–5.
139. DES VIGNES F, PIESMAN J, HEFFERNAN R, SCHULZE TL, STAFFORD KC 3RD, FISH D. Effect of tick removal on transmission of *Borrelia burgdorferi* and *Ehrlichia phagocytophila* by *Ixodes scapularis* nymphs. J Infect Dis. 2001;183(5):773-8.
140. DIAS JA, NÚNCIO MS, GONÇALVES A. [Contribuição para a elaboração de um inventário da fauna ixodoideológica (Acarina-Ixodoidea) de Portugal]. Garcia de Orta. 1994;20(1-2):49-68. [Article in Portuguese and abstract in English].
141. DOLAN MC, MAUPIN GO, SCHNEIDER BS, DENATALE C, HAMON N, COLE C, ZEIDNER NS, STAFFORD KC 3RD. Control of immature *Ixodes scapularis* (Acari: Ixodidae) on rodent reservoirs of *Borrelia burgdorferi* in a residential community of southeastern Connecticut. J Med Entomol. 2004;41(6):1043-54.
142. DONATIEN A, LESTOQUARD F. Existence en Algérie d'une *Rickettsia* du chien. Bull Soc Pathol Exot. 1935;28:418-9.
143. DONATIEN A, LESTOQUARD F. *Rickettsia bovis* Nouvelle espece pathogene pour le boeuf. Bull Soc Pathol Exot. 1936;29:1057-61.
144. DRANCOURT M, RAOULT D. Taxonomic position of the rickettsiae: current knowledge. FEMS Microbiol. 1994;13(1):13–24.
145. DRAZENOVICH N, FOLEY J, BROWN RN. Use of real-time quantitative PCR targeting the *msp2* protein gene to identify cryptic *Anaplasma phagocytophilum* infections in wildlife and domestic animals. Vector Borne Zoonotic Dis. 2006;6(1):83-90.
146. DUMLER JS. Molecular methods for ehrlichiosis and Lyme disease. Clin Lab Med. 2003;23(4):867-84.
147. DUMLER JS. *Anaplasma* and *Ehrlichia* infection. Ann NY Acad Sci. 2005;1063:361-73.
148. DUMLER JS, BAKKEN JS. Human granulocytic ehrlichiosis in Wisconsin and Minnesota: A frequent infection with the potential for persistence. J Infect Dis. 1996;173(4):1027-30.
149. DUMLER JS, BAKKEN JS. Human ehrlichioses: newly recognized infections transmitted by ticks. Annu Rev Med. 1998;49:201–13.
150. DUMLER JS, BAKKEN JS. Ehrlichial diseases of humans: emerging tick-borne infections. Clin Infect Dis. 1995;20(5):1102–10.
151. DUMLER JS, ASANOVICH KM, BAKKEN JS, RICHTER P, KIMSEY R, MADIGAN JE. Serologic cross-reactions among *Ehrlichia equi*, *Ehrlichia phagocytophila*, and human granulocytic *Ehrlichia*. J Clin Microbiol. 1995;33(5):1098–103.
152. DUMLER JS, BARBET AF, BEKKER CPJ, DASCH GA, PALMER GH, RAY SC, RIKIHISA Y, RURANGIRWA FR. Reorganization of genera in the families Rickettsiaceae and Anaplasmataceae in the order Rickettsiales: unification of some species of *Ehrlichia* with *Anaplasma*, *Cowdria* with *Ehrlichia* and *Ehrlichia* with *Neorickettsia*, description of six new species combinations and designation of *Ehrlichia equi* and 'HGE agent' as subjective synonyms of *Ehrlichia phagocytophila*. Int J Syst Evol Microbiol. 2001;51(Pt6):2145-65.
153. DUMLER JS, CHOI KS, GARCIA-GARCIA JC, BARAT NS, SCORPIO DG, GARYU JW, GRAB DJ, BAKKEN JS. Human granulocytic anaplasmosis and *Anaplasma phagocytophilum*. Emerg Infect Dis. 2005a;11(12):1828-34.
154. DUMLER JS, DOTEVALL L, GUSTAFSON R, GRANSTROM M. A population- based seroepidemiologic study of human granulocytic ehrlichiosis and Lyme borreliosis on the west coast of Sweden. J Infect Dis. 1997;175(3):720-2.
155. DUMLER JS, RIKIHISA Y, DASCH G. Genus I. *Anaplasma* Theiler 1910, 7 AL emend. Dumler, Barbet, Bekker, Dasch, Palmer, Ray, Rikihisa and Rurangirwa 2001, 2157. In: Bergey's Manual of Systematic Bacteriology. Garrity G, Brenner DJ, Krieg NR, Staley JR, editors. Volume II The Proteobacteria Part C The Alpha-, Beta-, Delta-, and Epsilonproteobacteria. 2nd edition. New-York: Spriger; 2005b. p. 117-25.
156. DUMLER JS, TRIGIANI ER, BAKKEN JS, AGUERO-ROSENFELD ME, WORMSER GP. Serum cytokine responses during acute human granulocytic ehrlichiosis. Clin Diagn Lab Immunol. 2000;7(1):6-8.
157. DUTTON JE, TODD JL. The nature of tick fever in the easthern part of the Congo Free State, with notes on the distribution and bionomics of the tick. Br Med J. 1905;2:1259-60.
158. ENGVALL EO, PETTERSSON B, PERSSON M, ARTURSSON K, JOHANSSON KE. A 16S rRNA-based PCR assay for detection and identification of granulocytic *Ehrlichia* species in dogs, horses, and cattle. J Clin Microbiol. 1996;34(9):2170-4.
159. ESTRADA-PENA A, JONGEJAN F. Ticks feeding on humans: a review of records on human-biting Ixodoidea with special reference to pathogen transmission. Exp Appl Acarol. 1999;23(9):685-715.
160. ESTRADA-PENA A, SANTOS-SILVA MM. The distribution of ticks (Acari: Ixodidae) of domestic livestock in Portugal. Exp Appl Acarol. 2005;36(3):233-46.
161. EWING SA, ROBERSON WR, BUCKNER RG, HAYAT BC. A new strain of *Ehrlichia canis*. J Am Vet Med Assoc. 1971;159(12):1771-4.
162. FANG QQ, MIXSON TR, HUGHES M, DUNHAM B, SAPP J. Prevalence of the agent of human granulocytic ehrlichiosis in *Ixodes scapularis* (Acari: Ixodidae) in the coastal southeastern United States. J Med Entomol. 2002;39(2):251-5.
163. FELEK S, TELFORD S 3RD, FALCO RC, RIKIHISA Y. Sequence analysis of p44 homologs expressed by *Anaplasma phagocytophilum* in infected ticks feeding on naive hosts and in mice infected by tick attachment. Infect Immun. 2004;72(2):659–66.

164. FENN K, CONLON C, JONES M, QUAIL MA, HOLROYD NE, PARKHILL J, BLAXTER M. Phylogenetic relationships of the *Wolbachia* of nematodes and arthropods. PLoS Pathog. 2006;2(10):e94.
165. FERQUEL E, GARNIER M, MARIE J, BERNEDE-BAUDUIN C, BARANTON G, PEREZ-EID C, POSTIC D. Prevalence of *Borrelia burgdorferi sensu lato* and Anaplasmataceae members in *Ixodes ricinus* ticks in Alsace, a focus of Lyme borreliosis endemicity in France. Appl Environ Microbiol. 2006;72(4):3074-8.
166. FERNANDEZ-SOTO P, PEREZ-SANCHEZ R, ENCINAS-GRANDES A. Molecular detection of *Ehrlichia phagocytophila* genogroup organisms in larvae of *Neotrombicula autumnalis* (Acari: Trombiculidae) captured in Spain. J Parasitol. 2001;87(6):1482-3.
167. FILIPE AR, REHACEK J, BACELLAR F, NÚNCIO MS. [*Ehrlichia canis* – Um "novo" agente de doença presente em Portugal, com importância em Saúde Pública]. Rev Port Doen Infec. 1990;4:235-8. [Article in Portuguese and anstract in English].
168. FINGERLE V, GOODMAN JL, JOHNSON RC, KURTTI TJ, MUNDERLOH UG, WILSKE B. Human granulocytic ehrlichiosis in southern Germany: increased seroprevalence in high-risk groups. J Clin Microbiol. 1997;35(12):3244–7.
169. FINGERLE V, MUNDERLOH UG, LIEGL G, WILSKE B. Coexistence of ehrlichiae of the phagocytophila group with *Borrelia burgdorferi* in *Ixodes ricinus* from Southern Germany. Med Microbiol Immunol (Berl). 1999;188(3):145-9.
170. FOGGIE A. Studies on the infectious agent of tick-borne fever in sheep. J Pathol Bacteriol. 1951;63:1–15.
171. FOLEY JE, BARLOUGH JE, KIMSEY RB, MADIGAN JE, DEROCK E, POLAND A. *Ehrlichia* spp. in cervids from California. J Wildl Dis. 1998;34(4):731-7.
172. FOLEY JE, CRAWFORD-MIKSZA L, DUMLER JS, GLASER C, CHAE JS, YEH E, SCHNURR D, HOOD R, HUNTER W, MADIGAN JE. Human granulocytic ehrlichiosis in northern California: two case descriptions with genetic analysis of the ehrlichiae. Clin Infect Dis. 1999a;29(2):388–92.
173. FOLEY JE, FOLEY P, JECKER M, SWIFT PK, MADIGAN JE. Granulocytic ehrlichiosis and tick infestation in mountain lions in California. J Wildl Dis. 1999b;35(4):703-9.
174. FOLEY JE, KRAMER V, WEBER D. Experimental infection of dusky-footed wood rats (*Neotoma fuscipes*) with *Ehrlichia phagocytophila sensu lato*. J Wildl Dis. 2002;38(1):194-8.
175. FOURNIER, PE, GRUNNENBERGER, F, JAULHAC, B, GASTINGER, RAOULT D. Evidence of *Rickettsia helvetica* infection in humans, eastern France. Emerg Infect Dis. 2000;6(4):389-92.
176. FRADIN MS. Mosquitoes and mosquito repellents: a clinician's guide. Ann Intern Med. 1998;128:931–40.
177. FRANCA I. [Infecção por espiroquetídeos do complexo *Borrelia burgdorferi* em Portugal. Perspectiva dermatológica e implicação da espécie *Borrelia lusitaniae*]. Tese de Doutoramento em Medicina, Universidade da Beira Interior, Covilhã, 2004, pp. 1-474. [PhD Thesis in Portuguese and abstract in English].
178. FRIEDMAN CS, ANDREE KB, BEAUCHAMP KA, MOORE JD, ROBBINS TT, SHIELDS JD, HEDRICK RP. '*Candidatus Xenohaliotis californiensis*', a newly described pathogen of abalone, *Haliotis* spp., along the west coast of North America. Int J Syst Evol Microbiol. 2000;50(Pt2):847–55.
179. FRITZ CL, BRONSON LR, SMITH CR, CRAWFORD-MIKSZA L, YEH E, SCHNURR D. Clinical, epidemiologic, and environmental surveillance for ehrlichiosis and anaplasmosis in an endemic area of northern California. J Vector Ecol. 2005;30(1):4-10.
180. FRITZ CL, KJEMTRUP AM, CONRAD PA, FLORES GR, CAMPBELL GL, SCHRIEFER ME, GALLO D, VUGIA DJ. Seroepidemiology of emerging tickborne infectious diseases in a Northern California community. J Infect Dis. 1997;175(6):1432-9.
181. GALLAGHER R, COLLINS S, TRUJILLO J, MCCREDIE K, AHEARN M, TSAI S, METZGAR R, AULAKH G, TING R, RUSCETTI F, GALLO R. Characterization of the continuous, differentiating myeloid cell line (HL-60) from a patient with acute promyelocytic leukaemia. Blood. 1979;54(3):713-33.
182. GARCIA JC, NUNEZ MJ, CASTRO B, FRAILE FJ, LOPEZ A, MELLA MC, BLANCO A, SIEIRA C, LOUREIRO E, PORTILLO A, OTEO JA. Human anaplasmosis: the first Spanish case confirmed by PCR. Ann NY Acad Sci. 2006;1078:545-7.
183. GARCIA-PEREZ AL, BARANDIKA J, OPORTO B, POVEDANO I, JUSTE RA. *Anaplasma phagocytophila* as an abortifacient agent in sheep farms from northern Spain. Ann NY Acad Sci. 2003;990:429-32.
184. GARDNER SL, HOLMAN RC, KREBS JW, BERKELMAN R, CHILDS JE. National surveillance for the human ehrlichioses in the United States, 1997-2001, and proposed methods for evaluation of data quality. Ann NY Acad Sci. 2003;990:80-9.
185. GARRITY GM, BELL JA, LILBURN TG. Taxonomic outline of the prokaryotes. In: Bergey's Manual of Systematic Bacteriology. Garrity GM, editor. 2nd edition. New-York: The Springer-Verlag; 2003. p. 47-8. Available from: http://141.150.157.80/bergeysoutline/main.htm.

186. GARRITY GM, BELL JA, LILBURN TG. Taxonomic outline of the prokaryotes. Family II. Anaplasmataceae. In: Bergey's Manual of Systematic Bacteriology. Garrity GM, editor. 2nd edition. New-York: The Springer-Verlag; 2004. p. 40-1. Available from: http://141.150.157.80/bergeysoutline/main.htm

187. GARRITY GM, JOHNSON KL, BELL JA, SEARLES DB. Taxonomic outline of the prokaryotes. In: Bergey's Manual of Systematic Bacteriology. Garrity GM, editor. 2nd edition. New-York: The Springer-Verlag; 2002b. p. 39-40. Available from: http://141.150.157.80/bergeysoutline/main.htm

188. GARRITY GM, WINTERS M, SEARLES DB. Taxonomic outline of the prokaryotes. In: Bergey's Manual of Systematic Bacteriology. Garrity GM, editor. 2nd edition. New-York: The Springer-Verlag; 2001. p. 29. Available from: http://141.150.157.80/bergeysoutline/main.htm

189. GARRITY GM, WINTERS M, KUO AW, SEARLES DB. Taxonomic outline of the prokaryotes. In: Bergey's Manual of Systematic Bacteriology Garrity GM, editor. 2nd edition. New-York: The Springer-Verlag; 2002a. p. 41-2. Available from: http://141.150.157.80/bergeysoutline/main.htm

190. GARYU JW, CHOI KS, GRAB DJ, DUMLER JS. Defective phagocytosis in *Anaplasma phagocytophilum*-infected neutrophils. Infect Immun. 2005;73(2):1187-90.

191. GE NL, KOCAN KM, BLOUIN EF, MURPHY GL. Developmental studies of *Anaplasma marginale* (Rickettsiales:Anaplasmataceae) in male *Dermacentor andersoni* (Acari:Ixodidae) infected as adults by using nonradioactive in situ hybridization and microscopy. J Med Entomol. 1996;33(6):911-20.

192. GE Y, YOSHIIE K, KURIBAYASHI F, LIN M, RIKIHISA Y. *Anaplasma phagocytophilum* inhibits human neutrophil apoptosis via upregulation of bfl-1, maintenance of mitochondrial membrane potential and prevention of caspase 3 activation. Cell Microbiol. 2005;7(1):29-38.

193. GERSHEL J. Human granulocytic ehrlichiosis presenting as abdominal pain. Pediatrics. 2000;106(3):602–4.

194. GOETHERT HK, TELFORD SR 3RD. Enzootic transmission of the agent of human granulocytic ehrlichiosis among cottontail rabbits. Am J Trop Med Hyg. 2003;68(6):633-7.

195. GOKCE HI, ROSS G, WOLDEHIWET Z. Inhibition of phagosome-lysosome fusion in ovine polymorphonuclear leucocytes by *Ehrlichia* (*Cytoecetes*) *phagocytophila*. J Comp Pathol. 1999;120(4):369-81.

196. GOODMAN JL, NELSON CM, KLEIN MB, HAYES SF, WESTON BW. Leukocyte infection by the granulocytic ehrlichiosis agent is linked to expression of a selectin ligand. J Clin Invest. 1999;103(3):407-12.

197. GOODMAN JL, NELSON C, VITALE B, MADIGAN JE, DUMLER JS, KURTTI TJ, MUNDERLOH UG. Direct cultivation of the causative agent of human granulocytic ehrlichiosis. N Engl J Med. 1996;334(4):209–15.

198. GORDON WS, BROWNLEE A, WILSON DR, MACLEOD J. "Tick-borne fever". A hitherto undescribed disease of sheep. J Comp Pathol Therap. 1932;65:301-7.

199. GRAY JS. Biology of *Ixodes* species ticks in relation to tick-borne zoonoses. Wien Klin Wochenschr. 2002;31;114(13-14):473-8.

200. GREIG B, ASANOVICH KM, ARMSTRONG PJ, DUMLER JS. Geographic, clinical, serologic, and molecular evidence of granulocytic ehrlichiosis, a likely zoonotic disease, in Minnesota and Wisconsin dogs. J Clin Microbiol. 1996;34(1):44-8.

201. GRIBBLE DH. Equine ehrlichiosis. J Am Vet Med Assoc. 1969;155: 462-9.

202. GROEN J, KORAKA P, NUR YA, AVSIC-ZUPANC T, GOESSENS WH, OTT A, OSTERHAUS AD. Serologic evidence of ehrlichiosis among humans and wild animals in The Netherlands. Eur J Clin Microbiol Infect Dis. 2002;21(1):46-9.

203. GRZESZCZUK A, STANCZAK J. Highly variable year-to-year prevalence of *Anaplasma phagocytophilum* in *Ixodes ricinus* ticks in northeastern Poland: a 4-year follow-up. Ann NY Acad Sci. 2006;1078:309-11.

204. GRZESZCZUK A, KARBOWIAK G, ZIARKO S, KOVALCHUK O. The root-vole *Microtus oeconomus* (Pallas, 1776): a new potential reservoir of *Anaplasma phagocytophilum*. Vector Borne Zoonotic Dis. 2006a;6(3):240-3.

205. GRZESZCZUK A, PUZANOWSKA B, MIEGOC H, PROKOPOWICZ D. Incidence and prevalence of infection with *Anaplasma phagocytophilum*. Prospective study in healthy individuals exposed to ticks. Ann Agric Environ Med. 2004a;11(1):155-7.

206. GRZESZCZUK A, STANCZAK J, KUBICA-BIERNAT B. Serological and molecular evidence of human granulocytic ehrlichiosis focus in the Bialowieza Primeval Forest (Puszcza Bialowieska), northeastern Poland. Eur J Clin Microbiol Infect Dis. 2002;21(1):6-11.

207. GRZESZCZUK A, STANCZAK J, KUBICA-BIERNAT B, RACEWICZ M, KRUMINIS-LOZOWSKA W, PROKOPOWICZ D. Human anaplasmosis in north-eastern Poland: seroprevalence in humans and prevalence in *Ixodes ricinus* ticks. Ann Agric Environ Med. 2004b;11(1):99-103.

208. GRZESZCZUK A, ZIARKO S, KOVALCHUK O, STANCZAK J. Etiology of tick-borne febrile illnesses in adult residents of North-Eastern Poland: report from a prospective clinical study.Int J Med Microbiol. 2006b;296(Suppl 40):242-9.

209. GUILLAUME B, HEYMAN P, LAFONTAINE S, VANDENVELDE C, DELMEE M, BIGAIGNON G. Seroprevalence of human granulocytic ehrlichiosis infection in Belgium. Eur J Clin Microbiol Infect Dis. 2002;21(5):397-400.
210. GUY E, TASKER S, JOYNSON DH. Detection of the agent of human granulocytic ehrlichiosis (HGE) in UK ticks using polymerase chain reaction. Epidemiol Infect. 1998;121(3):681-3.
211. HALASZ CL, NIEDT GW, KURTZ CP, SCORPIO DG, BAKKEN JS, DUMLER JS. A case of Sweet syndrome associated with human granulocytic anaplasmosis. Arch Dermatol. 2005;141(7):887-9.
212. HALOS L, VOURC'H G, COTTE V, GASQUI P, BARNOUIN J, BOULOUS HJ, VAYSSIER-TAUSSAT M. Prevalence of *Anaplasma phagocytophilum*, *Rickettsia* sp. and *Borrelia burgdorferi sensu lato* DNA in questing *Ixodes ricinus* ticks from France. Ann NY Acad Sci. 2006;1078:316-9.
213. HARDALO CJ, QUAGLIARELLO V, DUMLER JS. Human granulocytic ehrlichiosis in Connecticut: report of a fatal case. Clin Infect Dis. 1995;21(4):910-4.
214. HARVEY JW, SIMPSON CF, GASKIN JM. Cyclic thrombocytopenia induced by a *Rickettsia*-like agent in dogs. J Infect Dis 1978;137:182–8.
215. HARTELT K, OEHME R, FRANK H, BROCKMANN SO, HASSLER D, KIMMIG P. Pathogens and symbionts in ticks: prevalence of *Anaplasma phagocytophilum* (*Ehrlichia* sp.), *Wolbachia* sp., *Rickettsia* sp., and *Babesia* sp. in Southern Germany. Int J Med Microbiol. 2004;293(Suppl37):86-92.
216. HEIMER R, VAN ANDEL A, WORMSER GP, WILSON ML. Propagation of granulocytic *Ehrlichia* spp. from human and equine sources in HL-60 cells induced to differentiate into functional granulocytes. J Clin Microbiol. 1997;35(4):923-7.
217. HERMANOWSKA-SZPAKOWICZ T, SKOTARCZAK B, KONDRUSIK M, RYMASZEWSKA A, SAWCZUK M, MACIEJEWSKA A, ADAMSKA M, PANCEWICZ S, ZAJKOWSKA J. Detecting DNAs of *Anaplasma phagocytophilum* and *Babesia* in the blood of patients suspected of Lyme disease. Ann Agric Environ Med. 2004;11(2):351-4.
218. HERRON MJ, ERICSON ME, KURTTI TJ, MUNDERLOH UG. The interactions of *Anaplasma phagocytophilum*, endothelial cells, and human neutrophils. Ann NY Acad Sci. 2005;1063:374-82.
219. HERRON MJ, NELSON CM, LARSON J, SNAPP KR, KANSAS GS, GOODMAN JL. Intracellular parasitism by the human granulocytic ehrlichiosis bacterium through the P-selectin ligand, PSGL-1. Science. 2000;288(5471):1653-6.
220. HILDEBRANDT A, SCHMIDT KH, WILSKE B, DORN W, STRAUBE E, FINGERLE V. Prevalence of four species of *Borrelia burgdorferi sensu lato* and coinfection with *Anaplasma phagocytophila* in *Ixodes ricinus* ticks in Central Germany. Eur J Clin Microbiol Infect Dis. 2003;22(6):364-7.
221. HILTON E, DEVOTI J, BENACH JL, HALLUSKA ML,WHITE DJ, PAXTON H, DUMLER JS. Seroprevalence and seroconversion for tick-borne diseases in a high-risk population in the northeast United States. Am J Med. 1999;106(4):404–9.
222. HODZIC E, FISH D, MARETZKI CM, DE SILVA AM, FENG S, BARTHOLD SW. Acquisition and transmission of the agent of human granulocytic ehrlichiosis by *Ixodes scapularis* ticks. J Clin Microbiol. 1998;36(12):3574-8.
223. HOERAUF A, MAND S, ADJEI O, FLEISCHER B, BUTTNER DW. Depletion of *Wolbachia* endobacteria in *Onchocerca volvulus* by doxycycline and microfilaridermia after ivermectin treatment. Lancet. 2001;357(9266):1415–6.
224. HOLDEN K, BOOTHBY JT, ANAND S, MASSUNG RF. Detection of *Borrelia burgdorferi*, *Ehrlichia chaffeensis*, and *Anaplasma phagocytophilum* in ticks (Acari: Ixodidae) from a coastal region of California. J Med Entomol. 2003;40(4):534-9.
225. HOLDEN K, BOOTHBY JT, KASTEN RW, CHOMEL BB. Co-detection of *Bartonella henselae*, *Borrelia burgdorferi*, and *Anaplasma phagocytophilum* in *Ixodes pacificus* ticks from California, USA. Vector Borne Zoonotic Dis. 2006;6(1):99-102.
226. HOLDEN K, HODZIC E, FENG S, FREET KJ, LEFEBVRE RB, BARTHOLD SW. Coinfection with *Anaplasma phagocytophilum* alters *Borrelia burgdorferi* population distribution in C3H/HeN mice. Infect Immun. 2005;73(6):3440-4.
227. HOLLAND CJ, WEISS E, BURGDORFER W, COLE AI, KAKOMA I. *Ehrlichia risticii* sp. nov.: etiological agent of equine monocytic ehrlichiosis (synonym, Potomac horse fever). Int J Syst Bacteriol. 1985;35:524-6.
228. HOROWITZ HW, AGUERO-ROSENFELD M, DUMLER JS, MCKENNA DF, HSIEH TC, WU J, SCHWARTZ I, WORMSER GP. Reinfection with the agent of human granulocytic ehrlichiosis. Ann Intern Med. 1998a;129(6):461-3.
229. HOROWITZ HW, AGUERO-ROSENFELD ME, MCKENNA DF, HOLMGREN D, HSIEH TC, VARDE SA, DUMLER SJ, WU JM, SCHWARTZ I, RIKIHISA Y, WORMSER GP. Clinical and laboratory spectrum of culture proven human granulocytic ehrlichiosis: comparison with culture-negative cases. Clin Infect Dis. 1998b;27(5):1314-7.
230. HOROWITZ HW, HSIEH T-C, AGUERO-ROSENFELD ME, KALANTARPOUR F, CHOWDHURY I, WORMSER GP, WU JM. Antimicrobial susceptibility of *Ehrlichia phagocytophila.* Antimicrob Agents Chemother. 2001;45:786–8.

231. HOROWITZ HW, KILCHEVSKY E, HABER S, AGUERO-ROSENFELD M, KRANWINKEL R, JAMES EK, WONG SJ, CHU F, LIVERIS D, SCHWARTZ I. Perinatal transmission of the agent of human granulocytic ehrlichiosis. N Engl J Med. 1998c;339(6):375-8.

232. HOROWITZ HW, MARKS SJ, WEINTRAUB M, DUMLER JS. Brachial plexopathy associated with human granulocytic ehrlichiosis. Neurology. 1996;46(4):1026-9.

233. HOROWITZ HW, RAFFALLI J, NADELMAN RB, WU J, WORMSER GP. Saddleback fever due to human granulocytic ehrlichiosis. Lancet. 1998d;351(9103):650.

234. HOSSAIN D, AGUERO-ROSENFELD M, HOROWITZ HW, WU JM, HSIEH TC, SACHDEVA N, PETERSON SJ, DUMLER JS, WORMSER GP. Clinical and laboratory evolution of a culture-confirmed case of human granulocytic ehrlichiosis. Conn Med. 1999;63:265–70.

235. HOTOPP JC, LIN M, MADUPU R, CRABTREE J, ANGIUOLI SV, EISEN J, SESHADRI R, REN Q, WU M, UTTERBACK TR, SMITH S, LEWIS M, KHOURI H, ZHANG C, NIU H, LIN Q, OHASHI N, ZHI N, NELSON W, BRINKAC LM, DODSON RJ, ROSOVITZ MJ, SUNDARAM J, DAUGHERTY SC, DAVIDSEN T, DURKIN AS, GWINN M, HAFT DH, SELENGUT JD, SULLIVAN SA, ZAFAR N, ZHOU L, BENAHMED F, FORBERGER H, HALPIN R, MULLIGAN S, ROBINSON J, WHITE O, RIKIHISA Y, TETTELIN H. Comparative genomics of emerging human ehrlichiosis agents. PLoS Genet. 2006;2(2):e21.

236. HOUPIKIAN P, RAOULT D. Traditional and molecular techniques for the study of emerging bacterial diseases: one laboratory's perspective. Emerg Infect Dis. 2002;8(2):122-31.

237. HULINSKA D, VOTYPKA J, PLCH J, VLCEK E, VALESOVA M, BOJAR M, HULINSKY V, SMETANA K. Molecular and microscopical evidence of *Ehrlichia* spp. and *Borrelia burgdorferi sensu lato* in patients, animals and ticks in the Czech Republic. New Microbiol. 2002; 25(4):437-48.

238. HUNFELD K-P, BRADE V. Prevalence of antibodies against the human granulocytic ehrlichiosis agent in Lyme borreliosis patients from Germany. Eur J Clin Microbiol Infect Dis.1999;18(3):221–4.

239. IJDO JW, MUELLER AC. Neutrophil NADPH oxidase is reduced at the *Anaplasma phagocytophilum* phagosome. Infect Immun. 2004;72(9):5392-401.

240. IJDO JW, MEEK JI, CARTTER ML, MAGNARELLI LA, WU C, TENUTA SW, FIKRIG E, RYDER RW. The emergence of another tick-borne infection in the 12-town area around Lyme, Connecticut: human granulocytic ehrlichiosis. J Infect Dis. 2000;181(4):1388-93.

241. IJDO JW, SUN W, ZHANG Y, MAGNARELLI LA, FIKRIG E. Cloning of the gene encoding the 44-kilodalton antigen of the agent of human granulocytic ehrlichiosis and characterization of the humoral response. Infect Immun. 1998a;66(7):3264-9.

242. IJDO JW, ZHANG Y, ANDERSON ML, GOLDBERG D, FIKRIG E. Heat shock protein 70 of the agent of human granulocytic ehrlichiosis binds to *Borrelia burgdorferi* antibodies. Clin Diagn Lab Immunol. 1998b;5(1):118-20.

243. INOKUMA H, BROUQUI P, DRANCOURT M, RAOULT D. Citrate Synthase Gene Sequence: a New Tool for Phylogenetic Analysis and Identification of *Ehrlichia*. J Clin Microbiol. 2001b;39(9):3031-39.

244. INOKUMA H, TERADA Y, KAMIO T, RAOULT D, BROUQUI B. Analysis of the 16S rRNA Gene Sequence of *Anaplasma centrale* and Its Phylogenetic Relatedness to Other Ehrlichiae. Clin Diagn Lab Immunol 2001a;8(2):241–4.

245. JAHANGIR A, KOLBERT C, EDWARDS W, MITCHELL P, DUMLER JS, PERSING DH. Fatal pancarditis associated with human granulocytic ehrlichiosis in a 44-year-old man. Clin Infect Dis. 1998;27(6):1424-7.

246. JENKINS A, KRISTIANSEN BE, ALLUM AG, AAKRE RK, STRAND L, KLEVELAND EJ, VAN DE POL I, SCHOULS L. *Borrelia burgdorferi sensu lato* and *Ehrlichia* spp. in *Ixodes* ticks from southern Norway. J Clin Microbiol. 2001;39(10):3666-71.

247. KALANTARPOUR F, CHOWDHURY I, WORMSER GP, AGUERO-ROSENFELD ME. Survival of the human granulocytic ehrlichiosis agent under refrigeration conditions. J Clin Microbiol. 2000;38(6):2398-9.

248. KARLSSON U, BJOERSDORFF A, MASSUNG RF, CHRISTENSSON B. Human granulocytic ehrlichiosis--a clinical case in Scandinavia. Scand J Infect Dis. 2001;33(1):73-4.

249. KAWAHARA M, RIKIHISA Y, ISOGAI E, TAKAHASHI M, MISUMI H, SUTO C, SHIBATA S, ZHANG C, TSUJI M. Ultrastructure and phylogenetic analysis of '*Candidatus Neoehrlichia mikurensis*' in the family Anaplasmataceae, isolated from wild rats and found in *Ixodes ovatus* ticks. Int J Syst Evol Microbiol. 2004; 54(5):1837-43.

250. KAWAHARA M, RIKIHISA Y, LIN Q, ISOGAI E, TAHARA K, ITAGAKI A, HIRAMITSU Y, TAJIMA T. Novel Genetic Variants of *Anaplasma phagocytophilum*, *Anaplasma bovis*, *Anaplasma centrale*, and a Novel *Ehrlichia* sp. in Wild Deer and Ticks on Two Major Islands in Japan. Appl Environ Microbiol. 2006;72(2):1102-9.

251. KATAVOLOS P, ARMSTRONG P M, DAWSON J E, TELFORD S R III. Duration of tick attachment required for transmission of granulocytic ehrlichiosis. J Infect Dis. 1998;177(5):1422–5.

252. KIM CM, KIM MS, PARK MS, PARK JH, CHAE JS. Identification of *Ehrlichia chaffeensis*, *Anaplasma phagocytophilum*, and *A. bovis* in *Haemaphysalis longicornis* and *Ixodes persulcatus* ticks from Korea. Vector Borne Zoonotic Dis. 2003;3(1):17-26.

253. KIM CM, YI YH, YU DH, LEE MJ, CHO MR, DESAI AR, SHRINGI S, KLEIN TA, KIM HC, SONG JW, BAEK LJ, CHONG ST, O'GUINN ML, LEE JS, LEE IY, PARK JH, FOLEY J, CHAE JS. Tick-borne rickettsial pathogens in ticks and small mammals in Korea. Appl Environ Microbiol. 2006;72(9):5766-76.
254. KLEIN MB, HAYES SF, GOODMAN JL. Monocytic differentiation inhibits infection and granulocytic differentiation potentiates infection by the agent of human granulocytic ehrlichiosis. Infect Immun. 1998;66(7):3410-5.
255. KLEIN MB, HU S, CHAO CC, GOODMAN JL. The agent of human granulocytic ehrlichiosis induces the production of myelosuppressing chemokines without induction of proinflammatory cytokines. J Infect Dis. 2000;182 (1):200–5.
256. KLEIN MB, NELSON CM, GOODMAN JL. Antibiotic susceptibility of the newly cultivated agent of human granulocytic ehrlichiosis: promising activity of quinolones and rifamycins. Antimicrob Agents Chemother. 1997;41(1):76–9.
257. KOCAN KM, DE LA FUENTE J, BLOUIN EF, GARCIA-GARCIA JC. *Anaplasma marginale* (Rickettsiales: Anaplasmataceae): recent advances in defining host-pathogen adaptations of a tick-borne rickettsia. Parasitology. 2004;129(SupplS2):85-300.
258. KOLBERT C. Detection of the agent of human granulocytic ehrlichiosis by PCR. In: PCR protocols for emerging infectious diseases. HD. Persing HD editor. Washington: ASM Press; 1996. p. 106–11.
259. KOREN G, MATSUI D, BAILEY B. [DEET-based insect repellents: safety implications for children and pregnant and lactating women]. CMAJ. 2003;169:209–12. [Erratum in: CMAJ. 2003;169(4):283.]
260. KOWALSKI J, HOPFENMULLER W, FINGERLE V, MALBERG H, EISENBLATTER M, WAGNER J, MIKSITS K, HAHN H, IGNATIUS R. Seroprevalence of human granulocytic anaplasmosis in Berlin/Brandenburg, Germany: an 8-year survey. Clin Microbiol Infect. 2006;12(9):924-7.
261. KRAMER VL, RANDOLPH MP, HUI LT, IRWIN WE, GUTIERREZ AG, VUGIA DJ. Detection of the agents of human ehrlichioses in ixodid ticks from California. Am J Trop Med Hyg. 1999;60(1):62-5.
262. KRAUSE PJ, CORROW CL, BAKKEN JS. Successful treatment of human granulocytic ehrlichiosis in children using rifampin. Pediatrics. 2003; 112(3Pt1):e252–3.
263. KRAUSE PJ, MCKAY K, THOMPSON CA, SIKAND VK, LENTZ R, LEPORE T, CLOSTER L, CHRISTIANSON D, TELFORD SR, PERSING D, RADOLF JD, SPIELMAN A. Deer-Associated Infection Study Group. Disease-specific diagnosis of coinfecting tickborne zoonoses: babesiosis, human granulocytic ehrlichiosis, and Lyme disease. Clin Infect Dis. 2002;34(9):1184-91.
264. KRISTIANSEN BE, JENKINS A, TVETEN Y, KARSTEN B, LINE O, BJOERSDORFF A. [Human granulocytic ehrlichiosis in Norway]. Tidsskr Nor Laegeforen 2001;121(7):805-6. [Article in Norwegian and abstract in English].
265. LANE RS, FOLEY JE, EISEN L, LENNETTE ET, PEOT MA. Acarologic risk of exposure to emerging tick-borne bacterial pathogens in a semirural community in northern California. Vector Borne Zoonotic Dis. 2001;1(3):197-210.
266. LANE RS, MUN J, EISEN RJ, EISEN L. [Western gray squirrel (Rodentia: Sciuridae): a primary reservoir host of *Borrelia burgdorferi* in Californian oak woodlands?] J Med Entomol. 2005;42(3):388-96. [Erratum in: J Med Entomol. 2005;42(4):iv].
267. LANE RS, STEINLEIN DB, MUN J. Human behaviors elevating exposure to *Ixodes pacificus* (Acari: Ixodidae) nymphs and their associated bacterial zoonotic agents in a hardwood forest. J Med Entomol. 2004;41(2):239-48.
268. LAPPIN MR, BREITSCHWERDT EB, JENSEN WA, DUNNIGAN B, RHA JY, WILLIAMS CR, BREWER M, FALL M. Molecular and serologic evidence of *Anaplasma phagocytophilum* infection in cats in North America. J Am Vet Med Assoc. 2004;225(6):893-6.
269. LAFERL H, HOGREFE W, KOCK T, PICHLER H. A further case of acute human granulocytic ehrlichiosis in Slovenia. Eur J Clin Microbiol Infect Dis. 1999;18(5):385-6.
270. LEBECH A-M, HANSEN K, PANCHOLI P, SLOAN LM, MAGERA JM, PERSING DH. Immunological evidence of human granulocytic ehrlichiosis in Danish patients with Lyme neuroborreliosis. Scand J Infect Dis. 1998;30(2):173–6.
271. LEE FS, CHU FK, TACKLEY M, WU AD, ATRI A, WESSELS MR. Human granulocytic ehrlichiosis presenting as facial diplegia in a 42-year-old woman. Clin Infect Dis. 2000;31(5):1288-91.
272. LE FLÈCHE A, POSTIC D, GIRARDET K, PÉTER O, BARANTON G. Characterization of *Borrelia lusitaniae* sp. nov. by 16S ribosomal DNA sequence analysis. Int J Syst Bacteriol. 1997;47(4):921-5.
273. LEIBY DA, CHUNG AP, CABLE RG, TROUERN-TREND J, MCCULLOUGH J, HOMER MJ, REYNOLDS LD, HOUGHTON RL, LODES MJ, PERSING DH. Relationship between tick bites and the seroprevalence of *Babesia microti* and *Anaplasma phagocytophila* (previously *Ehrlichia* sp.) in blood donors. Transfusion. 2002;42(12):1585-91.
274. LEPIDI H, BUNNELL JE, MARTIN ME, MADIGAN JE, STUEN S, DUMLER JS. Comparative pathology and immunohistology associated with clinical illness after *Ehrlichia phagocytophila*-group infections. Am J Trop Med Hyg. 2000;62(1):29-37.

275. LESTER SJ, BREITSCHWERDT EB, COLLIS CD, HEGARTY BC. *Anaplasma phagocytophilum* infection (granulocytic anaplasmosis) in a dog from Vancouver Island. Can Vet J. 2005;46(9):825-7.

276. LESTOQUARD, F. Deuxieme note sur les Piroplasmoses du mouton en Algerie. L'anaplasmose: *Anaplasma ovis* nov. sp. Bulletin de la Societe de Pathologie Exotique. 1924;17:784-7.

277. LEUTENEGGER CM, PUSTERLA N, MISLIN CN, WEBER R, LUTZ H. Molecular evidence of coinfection of ticks with *Borrelia burgdorferi sensu lato* and the human granulocytic ehrlichiosis agent in Switzerland. J Clin Microbiol. 1999;37(10):3390-1.

278. LEVIN ML, FISH D. Acquisition of coinfection and simultaneous transmission of *Borrelia burgdorferi* and *Ehrlichia phagocytophila* by *Ixodes scapularis* ticks. Infect Immun. 2000;68(4):2183-6.

279. LEVIN ML, NICHOLSON WL, MASSUNG RF, SUMNER JW, FISH D. Comparison of the reservoir competence of medium-sized mammals and *Peromyscus leucopus* for *Anaplasma phagocytophilum* in Connecticut. Vector Borne Zoonotic Dis. 2002;2(3):125-36.

280. LIN M, RIKIHISA Y. *Ehrlichia chaffeensis* and *Anaplasma phagocytophilum* lack genes for lipid A biosynthesis and incorporate cholesterol for their survival. Infect Immun. 2003;71(9):5324-31.

281. LIN Q, ZHI N, OHASHI N, HOROWITZ HW, AGUERO-ROSENFELD ME, RAFFALLI J, WORMSER GP, RIKIHISA Y. Analysis of sequences and loci of p44 homologs expressed by *Anaplasma phagocytophila* in acutely infected patients. J Clin Microbiol. 2002;40(8): 2981–8.

282. LIZ JS, ANDERES L, SUMNER JW, MASSUNG RF, GERN L, RUTTI B, BROSSARD M. [PCR detection of granulocytic ehrlichiae in *Ixodes ricinus* ticks and wild small mammals in western Switzerland]. J Clin Microbiol. 2000;38(3):1002-7. [Erratum in: J Clin Microbiol. 2001;39(2):828].

283. LIZ JS, SUMNER JW, PFISTER K, BROSSARD M. PCR detection and serological evidence of granulocytic ehrlichial infection in roe deer (Capreolus *capreolus*) and chamois (*Rupicapra rupicapra*). J Clin Microbiol. 2002;40(3):892-7.

284. LO N, CASIRAGHI M, SALATI E, BAZZOCCHI C, BANDI C. How many wolbachia supergroups exist? Mol Biol Evol 2002;19(3):341-6.

285. LOPES DE CARVALHO I, NÚNCIO MS. Laboratory diagnosis of Lyme borreliosis in Portuguese National Institute of Heath (1990-2004). Eurosurveillance. 2006;11(10):257-260.

286. LOPES DE CARVALHO I, MILHANO N, SANTOS AS, ALMEIDA V, DE SOUSA R, NÚNCIO MS. Detection of *Borrelia lusitaniae*, *Rickettsia* IRS3 and IRS4 and *Anaplasma phagocytophilum* in *Ixodes ricinus* collected in Madeira Island, Portugal. In submission.

287. LOTRIC-FURLAN S, AVSIC-ZUPANC T, PETROVEC M, NICHOLSON WL, SUMNER JW, CHILDS JE, STRLE F. Clinical and serological follow-up of patients with human granulocytic ehrlichiosis in Slovenia. Clin Diagn Lab Immunol. 2001;8(5):899–903.

288. LOTRIC-FURLAN S, PETROVEC M, AVSIC-ZUPANC T, STRLE F. Clinical distinction between human granulocytic ehrlichiosis and the initial phase of tick-borne encephalitis. J Infect. 2000;40(1):55-8.

289. LOTRIC-FURLAN S, PETROVEC M, AVSIC-ZUPANC T, LOGAR M, STRLE F. Epidemiological, clinical and laboratory distinction between human granulocytic ehrlichiosis and the initial phase of tick-borne encephalitis.Wien Klin Wochenschr. 2002;114(13-14):636-40.

290. LOTRIC-FURLAN S, PETROVEC M, AVSIC-ZUPANC T, STRLE F. Concomitant tickborne encephalitis and human granulocytic ehrlichiosis.Emerg Infect Dis. 2005;11(3):485-8.

291. LOTRIC-FURLAN S, ROJKO T, PETROVEC M, AVSIC-ZUPANC T, STRLE F. Epidemiological, clinical and laboratory characteristics of patients with human granulocytic anaplasmosis in Slovenia. Wien Klin Wochenschr. 2006;118(21-22):708-13.

292. MACLEOD J, GORDON WS. Studies on tick-borne fever in sheep I: transmission by the tick *Ixodes ricinus* and descripition of the disease produced. Parasitology. 1933;25:273-83.

293. MADEWELL BR, GRIBBLE DH. Infection in two dogs with an agent resembling *Ehrlichia equi*. J Am Vet Med Assoc. 1982;180(5):512-4.

294. MADIGAN JE, BARLOUGH JE, DUMLER JS, SCHANKMAN NS, DEROCK E. Equine granulocytic ehrlichiosis in Connecticut caused by an agent resembling the human granulocytotropic ehrlichia. J Clin Microbiol. 1996;34(2):434-5.

295. MADIGAN JE, RICHTER PJ, KIMSEY RB, BARLOUGH JE, BAKKEN JS, DUMLER JS. Transmission and passage in horses of the agent ofhuman granulocytic ehrlichiosis. J Infect Dis. 1995;172(4):1141–4.

296. MADUREIRA ML, RAMALHINHO MG. In: [Notas sobre a distribuição, diagnose e ecologia dos Insectivora e Rodentia portugueses]. Faculdade de Ciências de Lisboa. Vol. XX, Arquivos do Museu Bocage. Lisboa: Publicação do Museu e Laboratório Zoológico e Antropológico; 1981. p. 165-263. [Book in Portuguese]

297. MAEDA K, MARKOWITZ N, HAWLEY RC, RISTIC M, COX D, MCDADE JE. Human infection with *Ehrlichia canis*, a leukocytic rickettsia. N Eng J Med. 1987;316(14):853-6.

298. MAGNARELLI LA, DUMLER JS, ANDERSON JF, JOHNSON RC, FIKRIG E. Coexistence of antibodies to tick-borne pathogens of babesiosis, ehrlichiosis, and Lyme borreliosis in human sera. J Clin Microbiol. 1995a;33(11):3054-7.

299. MAGNARELLI LA, IJDO JW, ANDERSON JF, PADULA SJ, FLAVELL RA, FIKRIG E. Human exposure to a granulocytic *Ehrlichia* and other tick-borne agents in Connecticut. J Clin Microbiol. 1998;36(10):2823-7.
300. MAGNARELLI LA, STAFFORD KC 3RD, MATHER TN, YEH MT, HORN KD, DUMLER JS. Hemocytic rickettsia-like organisms in ticks: serologic reactivity with antisera to ehrlichiae and detection of DNA of agent of human granulocytic ehrlichiosis by PCR. J Clin Microbiol. 1995b;33(10):2710-4.
301. MAKINEN J, VUORINEN I, OKSI J, PELTOMAA M, HE Q, MARJAMAKI M, VILJANEN MK. Prevalence of granulocytic *Ehrlichia* and *Borrelia burgdorferi sensu lato* in *Ixodes ricinus* ticks collected from Southwestern Finland and from Vormsi Island in Estonia. APMIS. 2003;111(2):355-62.
302. MANTELLI B, PECCHIOLI E, HAUFFE HC, ROSA R, RIZZOLI A. Prevalence of *Borrelia burgdorferi s.l.* and *Anaplasma phagocytophilum* in the wood tick *Ixodes ricinus* in the Province of Trento, Italy. Eur J Clin Microbiol Infect Dis. 2006;25(11):737-9.
303. MARQUEZ, FJ, MUNIAIN, MA, SORIGUER, RC, IZQUIERDO G, RODRÍGUEZ-BAÑO J, BOROBIO MV. Genotypic identification of an undescribed spotted fever group rickettsia in *Ixodes ricinus* from Southwestern Spain. Am J Trop Med Hyg. 1998;58(5):570-7.
304. MARTIN ME, BUNNELL JE, DUMLER JS. Pathology, immunohistology, and cytokine responses in early phases of human granulocytic ehrlichiosis in a murine model. J Infect Dis. 2000;181(1):374-8.
305. MARTIN ME, CASPERSEN K, DUMLER JS. Immunopathology and ehrlichial propagation are regulated by interferon-gamma and interleukin-10 in a murine model of human granulocytic ehrlichiosis. Am J Pathol. 2001;158(5):1881-8.
306. MASSUNG RF, SLATER KG. Comparison of PCR assays for detection of the agent of human granulocytic ehrlichiosis, *Anaplasma phagocytophilum*. J Clin Microbiol. 2003;41:717–22.
307. MASSUNG RF, COURTNEY JW, HIRATZKA SL, PITZER VE, SMITH G, DRYDEN RL. *Anaplasma phagocytophilum* in white-tailed deer. Emerg Infect Dis. 2005a;11(10):1604-6.
308. MASSUNG RF, LEVIN ML, MILLER NJ, MATHER TN. Reservoir Competency of Goats for *Anaplasma phagocytophilum*. Ann NY Acad Sci. 2006;1078:476-8.
309. MASSUNG RF, MATHER TN, PRIESTLEY RA, LEVIN ML Transmission efficiency of the AP-variant 1 strain of *Anaplasma phagocytophila*. Ann NY Acad Sci. 2003a;990:75-9.
310. MASSUNG RF, MAUEL MJ, OWENS JH, ALLAN N, COURTNEY JW, STAFFORD KC 3RD, MATHER TN. Genetic variants of *Ehrlichia phagocytophila*, Rhode Island and Connecticut. Emerg Infect Dis. 2002;8(5):467-72.
311. MASSUNG RF, OWENS JH, ROSS D, REED KD, PETROVEC M, BJOERSDORFF A, COUGHLIN RT, BELTZ GA, MURPHY CI. Sequence analysis of the ank gene of granulocytic ehrlichiae. J Clin Microbiol. 2000;38(8):2917-22.
312. MASSUNG RF, PRIESTLEY RA, MILLER NJ, MATHER TN, LEVIN ML. Inability of a variant strain of *Anaplasma phagocytophilum* to infect mice. J Infect Dis. 2003b;188(11):1757-63.
313. MASSUNG RF, SLATER K, OWENS JH, NICHOLSON WL, MATHER TN, SOLBERG VB, OLSON JG. Nested PCR assay for detection of granulocytic ehrlichiae. J Clin Microbiol. 1998;36(4):1090-5.
314. MASSUNG RF, ZEIDNER NS, DOLAN MC, ROELLIG D, GABITZSCH E, TROUGHTON DR, LEVIN ML. Prophylactic use of sustained-release doxycycline blocks tick-transmitted infection by *Anaplasma phagocytophilum* in a murine model. Ann NY Acad Sci. 2005b;1063:436-8.
315. MASTRANDREA S, MURA MS, TOLA S, PATTA C, TANDA A, PORCU R, MASALA G. Two cases of human granulocytic ehrlichiosis in Sardinia, Italy confirmed by PCR. Ann NY Acad Sci. 2006;1078:548-51.
316. MATUSCHKA FR, EIFFERT H, OHLENBUSCH A, RICHTER D, SCHEIN E, SPIELMAN A. Transmission of the agent of Lyme disease on a subtropical island. Trop Med Parasitol. 1994;45:39-44.
317. MATUSCHKA FR, KLUNG B, SCHINKEL T, SPIELMAN A, RICHTER D. Diversity of European Lyme disease spirochetes at the southern margin of their range. Appl Environ Microbiol. 1998;64(5):1980-2.
318. MAURIN M, ABERGEL C, RAOULT D. DNA gyrase-mediated natural resistance to fluoroquinolones in *Ehrlichia* spp. Antimicrob. Agents Chemother. 2001;45(7):2098–105.
319. MAURIN M, BAKKEN JS, DUMLER JS. Antibiotic susceptibilities of *Anaplasma* (*Ehrlichia*) *phagocytophilum* strains from various geographic areas in the United States. Antimicrob Agents Chemother. 2003;47(1):413–15.
320. MAYER-SCHOLL A, AVERHOFF P, ZYCHLINSKY A. How do neutrophils and pathogens interact? Curr Opin Microbiol. 2004;7(1):62-6.
321. MCDONALD D, BARRET P. In: [Mamíferos de Portugal e Europa]. Guias FAPAS, Fundo de protecção dos animais selvagens. Porto: Câmara Municipal do Porto, Pelouro do Ambiente; 1999. p. 1-315. [Book in Portuguese].
322. MCQUISTON JH, CHILDS JE, CHAMBERLAND ME, TABOR E. [Transmission of tick-borne agents of disease by blood transfusion: a review of known and potential risks in the United States]. Transfusion. 2000;40(3):274-84. [Erratum in: Transfusion 2000;40(7):891].

323. MCQUISTON JH, PADDOCK CD, HOLMAN RC, CHILDS JE. The human ehrlichioses in the United States. Emerg Infect Dis 1999;5(5):635-42.
324. MICHALSKI M, ROSENFIELD C, ERICKSON M, SELLE R, BATES K, ESSAR D, MASSUNG R. *Anaplasma phagocytophilum* in central and western Wisconsin: a molecular survey. Parasitol Res. 2006;99(6):694-9.
325. MISAO T, KOBAYASHI Y. Studies on infectious mononucleosis (glandular fever). I. isolation of etiologic agent from blood, bone marrow and lymph node of a patient with infectious mononucleosis by using mice. Kyushu J Med Sci. 1955;6:145-52.
326. MISIĆ-MAJERUS L, BUJIC N, MADARIC V, AVSIC-ZUPANC T, MILINKOVIC S. [Human anaplasmosis (ehrlichiosis)]. Acta Med Croatica 2006;60(5):411-9. [Article in Croatian and abstract in English].
327. MITCHELL PD, REED KD, HOFKES JM. Immunoserologic evidence of coinfection with *Borrelia burgdorferi*, *Babesia microti*, and human granulocytic *Ehrlichia* species in residents of Wisconsin and Minnesota. J Clin Microbiol. 1996;34(3):724-7.
328. MODI KS, DAHL DC, BERKSETH RO, SCHUT R, GREENO E. Human granulocytic ehrlichiosis presenting with acute renal failure and mimicking thrombotic thrombocytopenic purpura. A case report and review. Am J Nephrol. 1999;19(6):677-81.
329. MOROZOVA OV, DOBROTVORSKY AK, LIVANOVA NN, TKACHEV SE, BAKHVALOVA VN, BEKLEMISHEV AB, CABELLO FC. PCR detection of *Borrelia burgdorferi sensu lato*, tick-borne encephalitis virus, and the human granulocytic ehrlichiosis agent in *Ixodes persulcatus* ticks from Western Siberia, Russia. J Clin Microbiol. 2002;40(10):3802-4.
330. MOSS WJ, DUMLER JS. Simultaneous infection with *Borrelia burgdorferi* and human granulocytic ehrlichiosis. Pediatr Infect Dis J. 2003;22(1): 91–2.
331. MOULDER JW. Order I. Rickettsiales Gieszczykiewicz 1939. In: Bergey's Manual of Determinative Bacteriology. Buchanan RE, Gibbons NE editors, 8th edition. Baltimore: The Williams & Wilkins Co; 1974. p. 882-918.
332. MOTT J, BARNEWALL RE, RIKIHISA Y. Human granulocytic ehrlichiosis agent and *Ehrlichia chaffeensis* reside in different cytoplasmic compartments in HL-60 cells. Infect Immun. 1999;67(3):1368-78.
333. MOTT J, RIKIHISA Y. Human granulocytic ehrlichiosis agent inhibits superoxide anion generation by human neutrophils. Infect Immun. 2000;68(12):6697-703.
334. MUNDERLOH UG, JAURON SD, FINGERLE V, LEITRITZ L, HAYES SF, HAUTMAN JM, NELSON CM, HUBERTY BW, KURTTI TJ, AHLSTRAND GG, GREIG B, MELLENCAMP MA, GOODMAN JL. Invasion and intracellular development of the human granulocytic ehrlichiosis agent in tick cell culture. J Clin Microbiol. 1999;37(8):2518-24.
335. MUNDERLOH UG, LYNCH MJ, HERRON MJ, PALMER AT, KURTTI TJ, NELSON RD, GOODMAN JL. Infection of endothelial cells with *Anaplasma marginale* and *A. phagocytophilum*. Vet Microbiol. 2004;101(1):53-64.
336. MUNDERLOH UG, MADIGAN JE, DUMLER JS, GOODMAN JL, HAYES SF, BARLOUGH JE, NELSON CM, KURTTI TJ. Isolation of the equine granulocytic ehrlichiosis agent, *Ehrlichia equi*, in tick cell culture. J Clin Microbiol. 1996;34(3):664-70.
337. MURPHY CI, STOREY JR, RECCHIA J, DOROS-RICHERT LA, GINGRICH-BAKER C, MUNROE K, BAKKEN JS, COUGHLIN RT, BELTZ GA. Major antigenic proteins of the agent of human granulocytic ehrlichiosis are encoded by members of a multigene family. Infect Immun. 1998;66(8):3711-8.
338. NADELMAN RB, HOROWITZ HW, HSIEH T-C, WU JM, AGUERO-ROSENFELD ME, SCHARTZ I, NOWAKOWSKI J, VARDE S, WORMSER GP. Simultaneous human granulocytic ehrlichiosis and Lyme borreliosis. N Engl J Med. 1997;337:27-30.
339. NEEDHAM GR. Evaluation of five popular methods for tick removal. Pediatrics. 1985;75(6):997-1002.
340. NICHOLSON WL, CASTRO MB, KRAMER VL, SUMNER JW, CHILDS JE. [Dusky-footed wood rats (*Neotoma fuscipes*) as reservoirs of granulocytic ehrlichiae (Rickettsiales: Ehrlichieae) in northern California]. J Clin Microbiol. 1999;37(10):3323-7. [Erratum in: J Clin Microbiol 1999;37(12):4206].
341. NIETO NC, DABRITZ H, FOLEY P, DRAZENOVICH N, CALDER L, ADJEMIAN J, CONRAD PA, FOLEY JE. Ectoparasite diversity and exposure to vector-borne disease agents in wild rodents in central coastal California. J Med Entomol. 2007;44(2):328-35.
342. NÚNCIO MS. [Contribuição para o estudo de borrélias e borreliose de Lyme em Portugal]. Tese de Doutoramento, Faculdade de Ciências, Universidade de Lisboa, Lisboa, 2001. [PhD Thesis in Portuguese and abstract in English].
343. NÚNCIO MS, PÉTER O, ALVES MJ, BACELLAR F, FILIPE AR. [Isolamento e caracterização de borrélias de *Ixodes ricinus L.* em Portugal]. Rev Port Doen Infec 1993;16(3):175-9. [Article in Portuguese].
344. NÚNCIO MS, SCHOULS LM, VAN DE POL I, BACELLAR F, FILIPE A. The role of *Ixodes ricinus* as a vector of zoonotic agents in Madeira Island. In: Proceedings of the Seventh Bienal Meeting of European Tick Study Group, Oxford, United Kingdom, 2000 April 28.
345. NUTI M, SERAFINI DA, BASSETTI D, GHIONNI A, RUSSINO F, ROMBOLA P, MACRI G, LILLINI E. *Ehrlichia* infection in Italy. Emerg Infect Dis. 1998;4(4):663-5.

346. NYARKO E, GRAB DJ, DUMLER JS. *Anaplasma phagocytophilum*-infected neutrophils enhance transmigration of *Borrelia burgdorferi* across the human blood brain barrier *in vitro*. Int J Parasitol. 2006;36(5):601-5.
347. OEHME R, HARTELT K, BACKE H, BROCKMANN S, KIMMIG P. Foci of tick-borne diseases in southwest Germany. Int J Med Microbiol. 2002,291(Suppl. 33):22-9.
348. OGDEN NH, BOWN K, HORROCKS BK, WOLDEHIWET Z, BENNETT M. Granulocytic *Ehrlichia* infection in ixodid ticks and mammals in woodlands and uplands of the U.K. Med Vet Entomol. 1998;12(4):423-9.
349. OGDEN NH, MAAROUF A, BARKER IK, BIGRAS-POULIN M, LINDSAY LR, MORSHED MG, O'CALLAGHAN CJ, RAMAY F, WALTNER-TOEWS D, CHARRON DF. Climate change and the potential for range expansion of the Lyme disease vector *Ixodes scapularis* in Canada. Int J Parasitol. 2006;36(1):63-70.
350. OHASHI N, INAYOSHI M, KITAMURA K, KAWAMORI F, KAWAGUCHI D, NISHIMURA Y, NAITOU H, HIROI M, MASUZAWA T. *Anaplasma phagocytophilum*-infected ticks, Japan. Emerg Infect Dis. 2005;11(11):1780-3.
351. OHASHI N, ZHI N, LIN Q, RIKIHISA Y. Characterization and transcriptional analysis of gene clusters for a type IV secretion machinery in human granulocytic and monocytic ehrlichiosis agents. Infect Immun. 2002;70(4):2128-38.
352. O'NEILL SL, GIORDANO R, COLBERT AM, KARR TL, ROBERTSON HM. 16S rRNA phylogenetic analysis of the bacterial endosymbionts associated with cytoplasmic incompatibility in insects. Proc Nat Acad Sci USA. 1992;89:2699-702.
353. ONGUT G, OGUNC D, MUTLU G, COLAK D, GULTEKIN M, GUNSEREN F, DONMEZ L, TUNCER D. Seroprevalence of Antibodies to *Anaplasma phagocytophilum* in Antalya, Turkey. Infection. 2006;34(2):107-9.
354. OPORTO B, GIL H, BARRAL M, HURTADO A, JUSTE RA, GARCIA-PEREZ AL. A survey on *Anaplasma phagocytophila* in wild small mammals and roe deer (*Capreolus capreolus*) in Northern Spain. Ann NY Acad Sci. 2003;990:98-102.
355. OTEO JA, BLANCO JR, MARTINEZ DE ARTOLA V, IBARRA V. First report of human granulocytic ehrlichiosis from southern Europe (Spain). Emerg Infect Dis. 2000;6(4):430-2.
356. OTEO JA, GIL H, BARRAL M, PEREZ A, JIMENEZ S, BLANCO JR, MARTINEZ DE ARTOLA V, GARCIA-PEREZ A, JUSTE RA. Presence of granulocytic ehrlichia in ticks and serological evidence of human infection in La Rioja, Spain. Epidemiol Infect. 2001;127(2):353-8.
357. PADDOCK CD, CHILDS JE. *Ehrlichia chaffeensis*: a prototypical emerging pathogen. Clin Microbiol Rev. 2003;16(1):37-64.
358. PALMER GH, FUTSE JE, KNOWLES DP JR, BRAYTON KA. Insights into mechanisms of bacterial antigenic variation derived from the complete genome sequence of *Anaplasma marginale*. Ann NY Acad Sci. 2006;1078:15-25.
359. PANCHOLI P, KOLBERT CP, MITCHELL PD, REED KD JR, DUMLER JS, BAKKEN JS, TELFORD SR 3RD, PERSING DH. *Ixodes dammini* as a potential vector of human granulocytic ehrlichiosis. J Infect Dis. 1995;172(4):1007-12.
360. PARK J, CHOI KS, DUMLER JS. Major surface protein 2 of *Anaplasma phagocytophilum* facilitates adherence to granulocytes. Infect Immun. 2003a;71(7):4018-25.
361. PARK JH, HEO EJ, CHOI KS, DUMLER JS, CHAE JS. Detection of antibodies to *Anaplasma phagocytophilum* and *Ehrlichia chaffeensis* antigens in sera of Korean patients by western immunoblotting and indirect immunofluorescence assays. Clin Diagn Lab Immunol. 2003b;10(6):1059-64.
362. PARK J, KIM KJ, CHOI KS, GRAB DJ, DUMLER JS. *Anaplasma phagocytophilum* AnkA binds to granulocyte DNA and nuclear proteins. Cell Microbiol. 2004;6(8):743-51.
363. PAROLA P, RAOULT D. [Ticks and tickborne bacterial diseases in humans: an emerging infectious threat]. Clin Infect Dis. 2001a;32(6):897-928. [Erratum in: Clin Infect Dis. 2001;33(5):749].
364. PAROLA P, RAOULT D. Tick-borne bacterial diseases emerging in Europe. Clin Microbiol Infect. 2001b;7(2):80-3.
365. PEREZ M, BODOR M, ZHANG C, XIONG Q, RIKIHISA Y. Human infection with *Ehrlichia canis* accompanied by clinical signs in Venezuela. Ann NY Acad Sci. 2006;1078:110-7.
366. PEREZ M, RIKIHISA Y, WEN B. *Ehrlichia canis*-like agent isolated from a man in Venezuela: antigenic and genetic characterization. J Clin Microbiol. 1996;34(9):2133-9.
367. PETROVEC M, BIDOVEC A, SUMNER JW, NICHOLSON WL, CHILDS JE, AVSIC-ZUPANC T. Infection with *Anaplasma phagocytophila* in cervids from Slovenia: evidence of two genotypic lineages. Wien Klin Wochenschr. 2002;114(13-14):641-7.
368. PETROVEC M, LOTRIC FURLAN S, ZUPANC TA, STRLE F, BROUQUI P, ROUX V, DUMLER JS. Human disease in Europe caused by a granulocytic *Ehrlichia* species. J Clin Microbiol. 1997;35(6):1556-9.
369. PETROVEC M, SIXL W, SCHWEIGER R, MIKULASEK S, ELKE L, WUST G, MARTH E, STRASEK K, STUNZNER D, AVSIC-ZUPANC T. Infections of wild animals with *Anaplasma phagocytophila* in Austria and the Czech Republic. Ann NY Acad Sci. 2003;990:103-6.

370. PETROVEC M, SUMNER JW, NICHOLSON WL, CHILDS JE, STRLE F, BARLIC J, LOTRIC-FURLAN S, AVSIC ZUPANC T. Identity of ehrlichial DNA sequences derived from *Ixodes ricinus* ticks with those obtained from patients with human granulocytic ehrlichiosis in Slovenia. J Clin Microbiol. 1999;37(1):209-10.

371. PHILIP CB. Order I. Rickettsiales Buchanan and Buchanan 1938, emend. Gieszczykiewicz, 1939. In: Bergey's Manual of Determinative Bacteriology. Breed RS, Murray EGD, Smith NR editors. 7th edition. Baltimore: The Williams & Wilkins Co; 1957. p. 934-84.

372. PHILIP CB, HADLOW WJ, HUGHES LE. *Neorickettsia helminthoeca*, a new rickettsia-like disease agent in dogs in western United States transmitted by a helminth. In: Procedings of the VI International Congress on Microbiology; Roma , Italy; 1953; Vol II, p.256-7.

373. PICCOLIN G, BENEDETTI G, DOGLIONI C, LORENZATO C, MANCUSO S, PAPA N, PITTON L, RAMON MC, ZASIO C, BERTIATO G. A study of the presence of *B. burgdorferi*, *Anaplasma* (previously *Ehrlichia*) *phagocytophilum*, *Rickettsia*, and *Babesia* in *Ixodes ricinus* collected within the territory of Belluno, Italy. Vector Borne Zoonotic Dis. 2006;6(1):24-31.

374. PIERARD D, LEVTCHENKO L, DAWSON JE, LAUWERS S. Ehrlichiosis in Belgium. Lancet. 1995;346:1233-4.

375. POITOUT FM, SHINOZAKI JK, STOCKWELL PJ, HOLLAND CJ, SHUKLA SK. Genetic variants of *Anaplasma phagocytophilum* infecting dogs in Western Washington State. J Clin Microbiol. 2005;43(2):796-801.

376. POLIN H, HUFNAGL P, HAUNSCHMID R, GRUBER F, LADURNER G. Molecular evidence of *Anaplasma phagocytophilum* in *Ixodes ricinus* ticks and wild animals in Austria. J Clin Microbiol. 2004;42(5):2285-6.

377. POPOV VL, HAN VC, CHEN S-M, DUMLER JS, FENG H-M, ANDREADIS TG, TESH RB, WALTER DH. Ultrastructural differentiation of the genogroups in the genus *Ehrlichia*. J Med Microbiol. 1998;47(3):235-51.

378. PRETZMAN C, RALPH D, STOTHARD DR, FUERST PA, RIKIHISA Y. 16S rRNA Gene Sequence of *Neorickettsia helminthoeca* and its phylogenetic alignment with members of the genus *Ehrlichia*. Int J Syst Bacteriol. 1995;45(2):207-11.

379. PRUKK T, AINSALU K, LAJA E, AIGRO A. Human granulocytic ehrlichiosis in Estonia. Emerg Infect Dis. 2003;9(11):1499-500.

380. PUSTERLA N, HUDER JB, FEIGE K, LUTZ H. Identification of a granulocytic *Ehrlichia* strain isolated from a horse in Switzerland and comparison with other rickettsiae of the *Ehrlichia phagocytophila* genogroup. J Clin Microbiol. 1998a;36(7):2035-7.

381. PUSTERLA N, WEBER R, WOLFENSBERGER C, SCHAR G, ZBINDEN R, FIERZ W, MADIGAN JE, DUMLER JS, LUTZ H. Serological evidence of human granulocytic ehrlichiosis in Switzerland. Eur J Clin Microbiol Infect Dis. 1998b;17: 207–9.

382. PUSTERLA N, LEUTENEGGER CM, HUDER JB, WEBER R, BRAUN U, LUTZ H. Evidence of the human granulocytic ehrlichiosis agent in *Ixodes ricinus* ticks in Switzerland. J Clin Microbiol. 1999a;37(5):1332-4.

383. PUSTERLA N, PUSTERLA JB, BRAUN U, LUTZ H. Experimental cross-infections with *Ehrlichia phagocytophila* and human granulocytic ehrlichia-like agent in cows and horses. Vet Rec. 1999b;145(11):311-4.

384. PUSTERLA N, CHANG CC, CHOMEL BB, CHAE JS, FOLEY JE, DEROCK E, KRAMER VL, LUTZ H, MADIGAN JE. Serologic and molecular evidence of *Ehrlichia* spp. in coyotes in California. J Wildl Dis. 2000;36(3):494-9.

385. PUSTERLA N, ANDERSON RJ, HOUSE JK, PUSTERLA JB, DEROCK E, MADIGAN JE. Susceptibility of cattle to infection with *Ehrlichia equi* and the agent of human granulocytic ehrlichiosis. J Am Vet Med Assoc. 2001;218(7):1160-2.

386. QIU H, JUN HW, MCCALL JW. Pharmacokinetics, formulation, and safety of insect repellent N,N-diethyl-3-methylbenzamide (deet): a review. J Am Mosq Control Assoc. 1998;14(1):12-27.

387. RAMSEY AH, BELONGIA EA, GALE CM, DAVIS JP. Outcomes of treated human granulocytic ehrlichiosis cases. Emerg Infect Dis. 2002;8(4):398-401.

388. RAR VA, FOMENKO NV, DOBROTVORSKY AK, LIVANOVA NN, RUDAKOVA SA, FEDOROV EG, ASTANIN VB, MOROZOVA OV. Tickborne pathogen detection, Western Siberia, Russia. Emerg Infect Dis. 2005;11(11):1708-15.

389. REHACEK J, KAASERER B, URVOLGYI J, LUKACOVA M, KOVACOVA E, KOCIANOVA E. Isolation of *Coxiella burnetii* and of an unknown rickettsial organism from *Ixodes ricinus* ticks collected in Austria. Eur J Epidemiol. 1994;10(6):719-23.

390. REMY V, HANSMANN Y, DE MARTINO S, CHRISTMANN D, BROUQUI P. Human anaplasmosis presenting as atypical pneumonitis in France. Clin Infect Dis. 2003;37(6):846-8.

391. RICHTER PJ, KIMSEY RB, MADIGAN JE, BARLOUGH JE, DUMLER JS, BROOKS DL. *Ixodes pacificus* (Acari: Ixodidae) as a vector of *Ehrlichia equi* (Rickettsiales: Ehrlichieae). J Med Entomol. 1996;33(1):1–5.

392. RICKETTS HT. Some aspects of Rocky Mountain spotted fever as shown by recent investigations. Med Rec. 1909;16:843-55.

393. RIKIHISA Y. Mechanisms to create a safe haven by members of the family Anaplasmataceae. Ann NY Acad Sci. 2003;990:548-55.

394. RIKIHISA Y. New findings on members of the family Anaplasmataceae of veterinary importance. Ann NY Acad Sci. 2006a;1078:438-45.

395. RIKIHISA Y. Ehrlichia subversion of host innate responses. Curr Opin Microbiol. 2006b;9(1):95-101.

396. RIKIHISA Y, PERRY BD. Causative ehrlichial organisms in Potomac horse fever. Infect Immun. 1985;49(3):513-7.

397. RIKIHISA Y, KAWAHARA M, WEN B, KOCIBA G, FUERST P, KAWAMORI F, SUTO C, SHIBATA S, FUTOHASHI M. Westen immunoblot analysis of *Haemobartonella muris* and comparision of 16S rRNA gene sequences of *H. muris*, *H. felis*, and *Eperythrozoon suis*. J Clin Microbiol. 1997;35:823-9.

398. RIKIHISA Y, ZHANG C, CHRISTENSEN BM. Molecular Characterization of *Aegyptianella pullorum* (*Rickettsiales*, *Anaplasmataceae*). J Clin Microbiol. 2003;41(11):5294-7.

399. RISTIC M, KREIER JP. Family III. Anaplasmataceae Philip 1957, 980AL. In: Bergey's Manual of Systematic Bacteriology. Krieg NR, Holt JG, editors. Vol I. Baltimore: Williams & Wilkins; 1984. p. 719-29.

400. RODRIGUES PA, MACHADO J, CARDOSO O, BACELLAR F, FILIPE A, PROENÇA R. Ehrlichiose: caso clínico. In: Livro de resumos do 4º Congresso Nacional de Doenças Infecciosas, Lisboa, Portugal; 1998 Março 26. [Abstract in Portuguese]

401. RUSCIO M, CINCO M. Human granulocytic ehrlichiosis in Italy: first report on two confirmed cases. Ann N Y Acad Sci 2003; 990:350-2.

402. SALAFSKY B, HE YX, LI J, SHIBUYA T, RAMASWAMY K. Short report: study on the efficacy of a new long-acting formulation of N, N-diethyl-m-toluamide (DEET) for the prevention of tick attachment.Am J Trop Med Hyg. 2000;62(2):169-72.

403. SANOGO YO, ZEAITER Z, CARUSO G, MEROLA F, SHPYNOV S, BROUQUI P, RAOULT D. *Bartonella henselae* in *Ixodes ricinus* ticks (Acari: Ixodida) removed from humans, Belluno province, Italy. Emerg Infect Dis. 2003;9(3):329-32.

404. SANTINO I, DEL PIANO M, SESSA R, FAVIA G, IORI A. Detection of four *Borrelia burgdorferi* genospecies and first report of human granulocytic ehrlichiosis agent in *Ixodes ricinus* ticks collected in central Italy. Epidemiol Infect. 2002;129(1):93-7.

405. SANTINO I, CAMMARATA E, FRANCO S, GALDIERO F, OLIVA B, SESSA R, CIPRIANI P, TEMPERA G, DEL PIANO M. Multicentric study of seroprevalence of *Borrelia burgdorferi* and *Anaplasma phagocytophila* in high-risk groups in regions of central and southern Italy. Int J Immunopathol Pharmacol. 2004;17(2):219-23.

406. SANTOS AS. ["*Ehrlichia canis*" Contribuição para o estudo epidemiológico deste agente em Portugal]. Tese de Licenciatura em Engenharia Zootécnica, Universidade de Évora, Évora, 1997. [Thesis in Portuguese and abstract in English].

407. SARIH M, M'GHIRBI Y, BOUATTOUR A, GERN L, BARANTON G, POSTIC D. Detection and identification of *Ehrlichia* spp. in ticks collected in Tunisia and Morocco. J Clin Microbiol. 2005;43(3):1127-32.

408. SHAW SE, BINNS SH, BIRTLES RJ, DAY MJ, SMITHSON R, KENNY MJ. Molecular evidence of tick-transmitted infections in dogs and cats in the United Kingdom. Vet Rec. 2005;157(21):645-8.

409. SCAIFE H, WOLDEHIWET Z, HART CA, EDWARDS SW. *Anaplasma phagocytophilum* reduces neutrophil apoptosis *in vivo*. Infect Immun. 2003;71(4):1995-2001.

410. SCHAUBER EM, GERTZ SJ, MAPLE WT, OSTFELD RS. Coinfection of blacklegged ticks (Acari: Ixodidae) in Dutchess County, New York, with the agents of Lyme disease and human granulocytic ehrlichiosis. J Med Entomol. 1998;35(5):901-3.

411. SCHOULS LM, VAN DE POL I, RIJPKEMA SG, SCHOT CS. Detection and identification of *Ehrlichia*, *Borrelia burgdorferi sensu lato*, and *Bartonella* species in Dutch *Ixodes ricinus* ticks. J Clin Microbiol. 1999;37(7):2215-22.

412. SCHULZE TL, JORDAN RA, SCHULZE CJ, MIXSON T, PAPERO M. Relative encounter frequencies and prevalence of selected *Borrelia*, *Ehrlichia*, and *Anaplasma* infections in *Amblyomma americanum* and *Ixodes scapularis* (Acari: Ixodidae) ticks from central New Jersey. J Med Entomol. 2005;42(3):450-6.

413. SCORPIO DG, CASPERSEN K, OGATA H, PARK J, DUMLER JS. Restricted changes in major surface protein-2 (msp2) transcription after prolonged *in vitro* passage of *Anaplasma phagocytophilum*. BMC Microbiol. 2004a;8(4):1.

414. SCORPIO DG, AKKOYUNLU M, FIKRIG E, DUMLER JS. CXCR2 blockade influences *Anaplasma phagocytophilum* propagation but not histopathology in the mouse model of human granulocytic anaplasmosis. Clin Diagn Lab Immunol. 2004b;11(5):963-8.

415. SCORPIO DG, VON LOEWENICH FD, BOGDAN C, DUMLER JS. Innate immune tissue injury and murine HGA: tissue injury in the murine model of granulocytic anaplasmosis relates to host innate immune response and not pathogen load. Ann NY Acad Sci. 2005;1063:425-8.

416. SEKEYOVA Z, FOURNIER PE, REHACEK J, RAOULT D. Characterization of a new spotted fever group rickettsia detected in *Ixodes ricinus* (Acari: Ixodidae) collected in Slovakia. J Med Entomol. 2000;37(5):707-13.

417. SHEA KW, CALIO AJ, KLEIN NC, CUNHA BA. *Ehrlichia equi* infection associated with rhabdomyolysis. Clin Infect Dis. 1996;22(3):605.

418. SHPYNOV S, FOURNIER PE, RUDAKOV N, TARASEVICH I, RAOULT D. Detection of members of the genera *Rickettsia*, *Anaplasma*, and *Ehrlichia* in ticks collected in the Asiatic part of Russia. Ann NY Acad Sci. 2006;1078:378-83.

419. SHUKLA SK, ASWANI V, STOCKWELL PJ, REED KD. Contribution of Polymorphisms in *ankA*, *gltA*, and *groESL* in Defining Genetic Variants of *Anaplasma phagocytophilum*. J Clin Microbiol. 2007;45(7):2312-5.

420. SILVA LEITÃO JL. [Infestação Natural de Bovinos Portugueses pelo *Anaplama marginale* (Theiler, 1910)]. Separata do Repositório de Trabalhos do Laboratório Central de Patologia Veterinária 1943;5(2):15-7. [Article in Portuguese].

421. SILVEIRA CA. [Ehrlichiose canina. Estudo clínico de uma população animal, na região urbana e rural de Setúbal – Implicações em Saúde Pública e Saúde Pública Veterinária]. Tese de Mestrado em Saúde Pública Veterinária, Faculdade de Medicina Veterinária, Universidade Técnica de Lisboa, Lisboa 1992. [MSc Thesis in Portuguese and abstract in English].

422. SIMSER JA, PALMER AT, FINGERLE V, WILSKE B, KURTTI TJ, MUNDERLOH UG. *Rickettsia monacensis* sp. nov., a spotted fever group *Rickettsia* from ticks (*Ixodes ricinus*) collected in a European city park. Appl Environ Microbiol. 2002;68(9):4559-66.

423. SIXL W, PETROVEC M, MARTH E, WUST G, STUNZNER D, SCHWEIGER R, AVSIC-ZUPANC T. Investigation of *Anaplasma phagocytophila* infections in *Ixodes ricinus* and *Dermacentor reticulatus* ticks in Austria. Ann NY Acad Sci. 2003;990:94-7.

424. SKARPHEDINSSON S, SOGAARD P, PEDERSEN C. Seroprevalence of human granulocytic ehrlichiosis in high-risk groups in Denmark. Scand J Infect Dis. 2001;33(3):206-10.

425. SKARPHEDINSSON S, JENSEN PM, KRISTIANSEN K. Survey of tickborne infections in Denmark. Emerg Infect Dis. 2005;11(7):1055-61.

426. SKORACKI M, MICHALIK J, SKOTARCZAK B, RYMASZEWSKA A, SIKORA B, HOFMAN T, WODECKA B, SAWCZUK M. First detection of *Anaplasma phagocytophilum* in quill mites (Acari: Syringophilidae) parasitizing passerine birds. Microbes Infect. 2006;8(2):303-7.

427. SKOTARCZAK B, RYMASZEWSKA A, WODECKA B, SAWCZUK M. Molecular evidence of coinfection of *Borrelia burgdorferi sensu lato*, human granulocytic ehrlichiosis agent, and *Babesia microti* in ticks from northwestern Poland. J Parasitol. 2003;89(1):194-6.

428. SMITH T, KILBOURNE FL. Investigators into the nature, causation, and prevention of Texas or southern cattle fever. Bull Bur Anim Ind US Dep Agric. 1893;1:301.

429. SONENSHINE DE. In: Biology of ticks. Vol I. New York: Oxford University Press, Inc; 1991 p. 1-447.

430. SPITALSKA E, KOCIANOVA E. Agents of *Ehrlichia phagocytophila* group and other microorganisms co-infecting ticks in southwestern Slovakia. Acta Virol. 2002;46:49-50.

431. SPRINGER SA, ALTICE FL. Human immunodeficiency virus infection with human granulocytic ehrlichiosis complicated by symptomatic lactic acidosis. Clin Infect Dis. 2003;36(12):e162-4.

432. SRETER T, SRETER-LANCZ Z, SZELL Z, KALMAN D. *Anaplasma phagocytophilum*: an emerging tick-borne pathogen in Hungary and Central Eastern Europe. Ann Trop Med Parasitol. 2004;98(4):401-5.

433. SROKA J, CHMIELEWSKA-BADORA J, DUTKIEWICZ J. *Ixodes ricinus* as a potential vector of *Toxoplasma gondii*. Ann Agric Environ Med. 2003;10(1):121-3.

434. STANCZAK J, GRZESZCZUK A. Seroprevalence of *Anaplasma phagocytophilum* among forestry rangers in northern and northeastern Poland. Ann NY Acad Sci. 2006;1078:89-91.

435. STANCZAK J, GABRE RM, KRUMINIS-LOZOWSKA W, RACEWICZ M, KUBICA-BIERNAT B. *Ixodes ricinus* as a vector of *Borrelia burgdorferi sensu lato*, *Anaplasma phagocytophilum* and *Babesia microti* in urban and suburban forests. Ann Agric Environ Med. 2004;11(1):109-14.

436. STANCZAK J, RACEWICZ M, KRUMINIS-LOZOWSKA W, KUBICA-BIERNAT B. Coinfection of *Ixodes ricinus* (Acari: Ixodidae) in northern Poland with the agents of Lyme borreliosis (LB) and human granulocytic ehrlichiosis (HGE). Int J Med Microbiol. 2002;291(Suppl 33):198-201.

437. STAFFORD KC 3RD, MASSUNG RF, MAGNARELLI LA, IJDO JW, ANDERSON JF. Infection with agents of human granulocytic ehrlichiosis, Lyme disease, and babesiosis in wild white-footed mice (*Peromyscus leucopus*) in Connecticut. J Clin Microbiol. 1999;37(9):2887-92.

438. STANNARD AA, GRIBBLE DH, SMITH RS. Equine ehrlichiosis. A disease with similarities to tick-borne fever and bovine petenchial fever. Vet Rec. 1969;84:149-50.

439. STEINER FE, PINGER RR, VANN CN, ABLEY MJ, SULLIVAN B, GRINDLE N, CLAY K, FUQUA C. Detection of *Anaplasma phagocytophilum* and *Babesia odocoilei* DNA in *Ixodes scapularis* (Acari: Ixodidae) collected in Indiana. J Med Entomol. 2006;43(2):437-42.

440. STOREY JR, DOROS-RICHERT LA, GINGRICH-BAKER C, MUNROE K, MATHER TN, COUGHLIN RT, BELTZ GA, MURPHY CI. Molecular cloning and sequencing of three granulocytic *Ehrlichia* genes encoding high-molecular-weight immunoreactive proteins. Infect Immun. 1998;66(4):1356-63.

441. STRLE F. Human granulocytic ehrlichiosis in Europe. Int J Med Microbiol. 2004;293(Suppl37):27-35.

442. STUEN S, BERGSTROM K, PALMER E. Reduced weight gain due to subclinical *Anaplasma phagocytophilum* (formerly *Ehrlichia phagocytophila*) infection. Exp Appl Acarol. 2002a28(1-4):209-15.

443. STUEN S, BERGSTROM K, PETROVEC M, VAN DE POL I, SCHOULS LM. Differences in clinical manifestations and hematological and serological responses after experimental infection with genetic variants of *Anaplasma phagocytophilum* in sheep. Clin Diagn Lab Immunol. 2003a;10(4):692-5.

444. STUEN S, DAHL H, BERGSTROM K, MOUM T. Unidirectional suppression of *Anaplasma phagocytophilum* genotypes in infected lambs. Clin Diagn Lab Immunol. 2005;12(12):1448-50.

445. STUEN S, ENGVALL EO, VAN DE POLL I, SCHOULS LM. Granulocytic ehrlichiosis in a roe deer calf in Norway. J Wildl Dis. 2001;37(3):614-6.

446. STUEN S, NEVLAND S, MOUM T. Fatal cases of Tick-borne fever (TBF) in sheep caused by several 16S rRNA gene variants of *Anaplasma phagocytophilum*. Ann NY Acad Sci. 2003b;990:433-4.
447. STUEN S, VAN DE POL I, BERGSTROM K, SCHOULS LM. Identification of *Anaplasma phagocytophila* (formerly *Ehrlichia phagocytophila*) variants in blood from sheep in Norway. J Clin Microbiol. 2002b;40(9):3192-7.
448. SUKUMARAN B, NARASIMHAN S, ANDERSON JF, DEPONTE K, MARCANTONIO N, KRISHNAN MN, FISH D, TELFORD SR, KANTOR FS, FIKRIG E. An *Ixodes scapularis* protein required for survival of *Anaplasma phagocytophilum* in tick salivary glands. J Exp Med. 2006;203(6):1507-17.
449. SUMNER JW, NICHOLSON WL, MASSUNG RF. PCR amplification and comparison of nucleotide sequences from the *groESL* heat shock operon of *Ehrlichia* species. J Clin Microbiol. 1997;35(8):2087-92.
450. SUMNER JW, STORCH GA, BULLER RS, LIDDELL AM, STOCKHAM SL, RIKIHISA Y, MESSENGER S, PADDOCK CD. PCR Amplification and Phylogenetic Analysis of *groESL* Operon Sequences from *Ehrlichia ewingii* and *Ehrlichia muris.* J Clin Microbiol. 2000;38(7):12746-9.
451. SWANSON SJ, NEITZEL D, REED KD, BELONGIA EA. Coinfections acquired from *Ixodes* ticks. Clin Microbiol Rev. 2006;19(4):708-27.
452. TATE CM, MEAD DG, LUTTRELL MP, HOWERTH EW, DUGAN VG, MUNDERLOH UG, DAVIDSON WR. Experimental infection of white-tailed deer with *Anaplasma phagocytophilum*, etiologic agent of human granulocytic anaplasmosis. J Clin Microbiol 2005;43(8):3595-601.
453. TAYLOR MJ, MAKUNDE WH, MCGARRY HF, TURNER JD, MAND S, HOERAUF A. Macrofilaricidal activity after doxycycline treatment of *Wuchereria bancrofti*: a double-blind, randomised placebo-controlled trial. Lancet. 2005;365(9477):2116-21.
454. TELFORD III SR, LEPORE TJ, SNOW P, WARNER CK, DAWSON JE. Human granulocytic ehrlichiosis in Massachusetts. Ann Intern Med. 1995;123:277-9.
455. TELFORD SR 3RD, DAWSON JE, KATAVOLOS P, WARNER CK, KOLBERT CP, PERSING DH. Perpetuation of the agent of human granulocytic ehrlichiosis in a deer tick-rodent cycle. Proc Natl Acad Sci USA. 1996;93(12):6209-14.
456. THEILER A. *Anaplasma marginale* (genus nov. et species nov.). The marginal points in the blood of cattle suffering from a specific disease. In: Repot of the Government, Transvaal. South Africa: Veterinary Bacteriology, Dept of Agriculture; 1910. p. 7-64.
457. THEILER, A. Further investigations into anaplasmosis of South African cattle. In: First Report of the Director of Veterinary Research. Union of South Africa; 1911. p. 7-46.
458. THOMAS V, ANGUITA J, BARTHOLD SW, FIKRIG E. Coinfection with *Borrelia burgdorferi* and the agent of human granulocytic ehrlichiosis alters murine immune responses, pathogen burden, and severity of Lyme arthritis. Infect Immun. 2001;69(5):3359-71.
459. THOMAS V, SAMANTA S, WU C, BERLINER N, FIKRIG E. *Anaplasma phagocytophilum* modulates gp91phox gene expression through altered interferon regulatory factor 1 and PU.1 levels and binding of CCAAT displacement protein. Infect Immun. 2005;73(1):208-18.
460. TYLEWSKA-WIERZBANOWSKA S, CHMIELEWSKI T, KONDRUSIK M, HERMANOWSKA-SZPAKOWICZ T, SAWICKI W, SULEK K. First cases of acute human granulocytic ehrlichiosis in Poland. Eur J Clin Microbiol Infect Dis. 2001;20(3):196-8.
461. TOMASIEWICZ K, MODRZEWSKA R, BUCZEK A, STANCZAK J, MACIUKAJC J. The risk of exposure to *Anaplasma phagocytophilum* infection in Mid-Eastern Poland. Ann Agric Environ Med. 2004;11(2):261-4.
462. TOPOLOVEC J, PUNTARIC D, ANTOLOVIC-POZGAIN A, VUKOVIC D, TOPOLOVEC Z, MILAS J, DRUSKO-BARISIC V, VENUS M. Serologically detected "new" tick-borne zoonoses in eastern Croatia Croat Med J. 2003;44(5):626-9.
463. TOZON N, PETROVEC M, AVSIC-ZUPANC T. Clinical and laboratory features of the first detected cases of *A. phagocytophila* infections in dogs from Slovenia. Ann NY Acad Sci. 2003;990:424-8.
464. TROFE J, REDDY KS, STRATTA RJ, FLAX SD, SOMERVILLE KT, ALLOWAY RR, EGIDI MF, SHOKOUH-AMIRI MH, GABER AO. Human granulocytic ehrlichiosis in pancreas transplant recipients. Transpl Infect Dis. 2001;3(1):34-9.
465. UHAA IJ, MACLEAN JD, GREENE CR, FISHBEIN DB. A case of human ehrlichiosis acquired in Mali: clinical and laboratory findings. Am J Trop Med Hyg. 1992;46:161–4.
466. UNVER A, FELEK S, PADDOCK CD, ZHI N, HOROWITZ HW, WORMSER GP, CULLMAN LC, RIKIHISA Y. Western blot analysis of sera reactive to human monocytic ehrlichiosis and human granulocytic ehrlichiosis agents. J Clin Microbiol. 2001a;39(11):3982-6.
467. UNVER A, PEREZ M, ORELLANA N, HUANG H, RIKIHISA Y. Molecular and antigenic comparison of *Ehrlichia canis* isolates from dogs, ticks, and a human in Venezuela. J Clin Microbiol. 2001b;39(8):2788-93.
468. VAN DOBBENBURGH A, VAN DAM AP, FIKRIG E. Human granulocytic ehrlichiosis in western Europe. N Engl J Med. 1999;340(15):1214-6.
469. VANNORSDALL MD, THOMAS S, SMITH RP, ZIMMERMAN R, CHRISTMAN R, VELLA JP. Human granulocytic ehrlichiosis in a renal allograft recipient: review of the clinical spectrum of disease in solid organ transplant patients. Transpl Infect Dis. 2002;4(2):97-101.
470. VARDE S, BECKLEY J, SCHWARTZ I. Prevalence of tick-borne pathogens in *Ixodes scapularis* in a rural New Jersey County. Emerg Infect Dis. 1998;4(1):97-9.

471. VON LOEWENICH FD, BAUMGARTEN BU, SCHROPPEL K, GEISSDORFER W, ROLLINGHOFF M, BOGDAN C. High diversity of *ankA* sequences of *Anaplasma phagocytophilum* among *Ixodes ricinus* ticks in Germany. J Clin Microbiol. 2003a;41(11):5033-40.

472. VON LOEWENICH FD, SCORPIO DG, REISCHL U, DUMLER JS, BOGDAN C. Frontline: control of *Anaplasma phagocytophilum*, an obligate intracellular pathogen, in the absence of inducible nitric oxide synthase, phagocyte NADPH oxidase, tumor necrosis factor, Toll-like receptor (TLR)2 and TLR4, or the TLR adaptor molecule MyD88. Eur J Immunol. 2004;34(7):1789-97.

473. VON LOEWENICH FD, STUMPF G, BAUMGARTEN BU, ROLLINGHOFF M, DUMLER JS, BOGDAN C. A case of equine granulocytic ehrlichiosis provides molecular evidence for the presence of pathogenic *Anaplasma phagocytophilum* (HGE agent) in Germany. Eur J Clin Microbiol Infect Dis. 2003b;22(5):303-5.

474. WALDER G, FALKENSAMMER B, AIGNER J, TIWALD G, DIERICH MP, WURZNER R, LECHLEITNER P. First documented case of human granulocytic ehrlichiosis in Austria. Wien Klin Wochenschr. 2003a;115(7-8):263-6.

475. WALDER G, FUCHS D, SARCLETTI M, BEREK K, FALKENSAMMER B, HUBER K, PETROVEC M, DIERICH MP, WURZNER R. Human granulocytic anaplasmosis in Austria: epidemiological, clinical, and laboratory findings in five consecutive patients from Tyrol, Austria. Int J Med Microbiol. 2006a;296(Suppl40):297-301.

476. WALDER G, LKHAMSUREN E, SHAGDAR A, BATAA J, BATMUNKH T, ORTH D, HEINZ FX, DANICHOVA GA, KHASNATINOV MA, WURZNER R, DIERICH MP. Serological evidence for tick-borne encephalitis, borreliosis, and human granulocytic anaplasmosis in Mongolia. Int J Med Microbiol. 2006b;296(Suppl40):69-75.

477. WALDER G, TIWALD G, DIERICH MP, WURZNER R. Serological evidence for human granulocytic ehrlichiosis in Western Austria. Eur J Clin Microbiol Infect Dis. 2003b;22(9):543-7.

478. WALLACE BJ, BRADY G, ACKMAN DM, WONG SJ, JACQUETTE G, LLOYD EE, BIRKHEAD GS. Human granulocytic ehrlichiosis in New York. Arch Intern Med. 1998;158(7):769-73.

479. WALKER DH, DUMLER JS. Emergence of the ehrlichioses as human health problems. Emerging Infect Dis. 1996;2:18–29.

480. WALKER DH, DUMLER JS. Human monocytic and granulocytic ehrlichioses. Discovery and diagnosis of emerging tick-borne infections and the critical role of the pathologist. Arch Pathol Lab Med. 1997;121(8):785-91.

481. WALKER DH, BAKKEN J, BROUQUI P, CHILDS J, CULLMAN L, DAWSON J, DUMLER S, FINGERLE V, GOODMAN J, HOGAN P, HOGREFE W, LIZ J, MAGNARELLI LA, MARKLEY J, MASSUNG R, MASTERS EJ, MURPHY C, OLANO J, PADDOCK C, PETROVEC M, RIKIHISA Y, STANDAERT S, WORMSER GP, YU X-J, AVSIC-ZUPANC T. Diagnosing human ehrlichioses: current status and recommendations. ASM News. 2000;66(5):287-90.

482. WALLS JJ, AGUERO-ROSENFELD M, BAKKEN JS, GOODMAN JL, HOSSAIN D, JOHNSON RC, DUMLER JS. Inter- and intralaboratory comparison of *Ehrlichia equi* and human granulocytic ehrlichiosis (HGE) agent strains for serodiagnosis of HGE by the immunofluorescent-antibody test. J Clin Microbiol. 1999;37(9):2968-73.

483. WALLS JJ, CATUREGLI P, BAKKEN JS, ASANOVICH KM, DUMLER JS. Improved sensitivity of PCR for diagnosis of human granulocytic ehrlichiosis using *epank1* genes of *Ehrlichia phagocytophila*-group ehrlichiae. J Clin Microbiol. 2000;38(1):354-6.

484. WALLS JJ, GREIG B, NEITZEL DF, DUMLER JS. Natural infection of small mammal species in Minnesota with the agent of human granulocytic ehrlichiosis. J Clin Microbiol. 1997;35(4):853-5.

485. WANG T, MALAWISTA SE, PAL U, GREY M, MEEK J, AKKOYUNLU M, THOMAS V, FIKRIG E. Superoxide anion production during *Anaplasma phagocytophila* infection. J Infect Dis. 2002;186(2):274-80.

486. WEBSTER P, IJDO JW, CHICOINE LM, FIKRIG E. The agent of human granulocytic ehrlichiosis resides in an endosomal compartment. J Clin Invest. 1998;101(9):1932-41.

487. WEISBURG WG, DOBSON ME, SAMUEL JE, DASCH GA, MALLAVIA LP, BACA O, MANDELCO L, SECHREST JE, WEISS E, WOESE CR. Phylogenetic diversity of the Rickettsiae. J Bacteriol.1989;171(8):4202-6.

488. WEISBURG WG, BARNS SM, PELLETIER DA, LANE DJ. 16S ribosomal DNA amplification for phylogenetic study. J Bacteriol. 1991;173(2):697-703.

489. WEISS E, MOULDER JW. Order I. Rickettsiales Gieszczkiewicz, 1939. In: Bergey's Manual of Sistematic Bacteriology. Krieg NR, Holt JG, editors. Vol I. 1st edition. Baltimore: The Williams & Wilkins; 1984. p. 687-729.

490. WEN B, CAO W, PAN H. Ehrlichiae and ehrlichial diseases in China. Ann NY Acad Sci. 2003;990:45-53.

491. WEN B, RIKIHISA Y, MOTT J, FUERST PA, KAWAHARA M, SUTO C. *Ehrlichia muris* sp. nov., identified on the basis of 16S rRNA base sequences and serological, morphological, and biological characteristics. Int J Syst Bacteriol. 1995;45(2):250-4.

492. WEN B, RIKIHISA Y, YAMAMOTO S, KAWABATA N, FUERST PA. Characterization of the SF Agent, an *Ehrlichia* sp. Isolated from the Fluke *Stellantchasmus falcatus*, by 16S rRNA Base Sequence, Serological, and Morphological Analyses. Int J Syst Bacteriol. 1996;46(1):149-54.

493. WITTESJO B, BJOERSDORFF A, ELIASSON I, BERGLUND J. First long-term study of the seroresponse to the agent of human granulocytic ehrlichiosis among residents of a tick-endemic area of Sweden. Eur J Clin Microbiol Infect Dis. 2001;20(3):173-8.
494. WOESSNER R, GAERTNER BC, GRAUER MT, WEBER K, MUELLER-LANTZSCH N, HUNFELD KP, TREIB J. Incidence and prevalence of infection with human granulocytic ehrlichiosis agent in Germany. A prospective study in young healthy subjects. Infection. 2001;29(5):271-3.
495. WONG SJ, BRADY GS, DUMLER JS. Serological responses to *Ehrlichia equi*, *Ehrlichia chaffeensis*, and *Borrelia burgdorferi* in patients from New York State. J Clin Microbiol. 1997;35(9):2198-205.
496. WORMSER GP, DATTWYLER RJ, SHAPIRO ED, HALPERIN JJ, STEERE AC, KLEMPNER MS, KRAUSE PJ, BAKKEN JS, STRLE F, STANEK G, BOCKENSTEDT L, FISH D, DUMLER JS, NADELMAN RB. The clinical assessment, treatment, and prevention of Lyme disease, human granulocytic anaplasmosis, and babesiosis: clinical practice guidelines by the Infectious Diseases Society of America. Clin Infect Dis. 2006a;43(9):1089-134.
497. WORMSER GP, FILOZOV A, TELFORD SR, UTPAT S, KAMER RS, LIVERIS D, WANG G, ZENTMAIER L, SCHWARTZ I, AGUERO-ROSENFELD ME. Dissociation between inhibition and killing by levofloxacin in human granulocytic anaplasmosis. Vector Borne Zoonotic Dis. 2006b;6(4):388-94.
498. YABSLEY MJ, ROMINES J, NETTLES VF. Detection of *Babesia* and *Anaplasma* species in rabbits from Texas and Georgia, USA. Vector Borne Zoonotic Dis. 2006;6(1):7-13.
499. YEH MT, MATHER TN, COUGHLIN RT, GINGRICH-BAKER C, SUMNER JW, MASSUNG RF. Serologic and molecular detection of granulocytic ehrlichiosis in Rhode Island. J Clin Microbiol. 1997;35(4):944-7.
500. YOSHIIE K, KIM HY, MOTT J, RIKIHISA Y. Intracellular infection by the human granulocytic ehrlichiosis agent inhibits human neutrophil apoptosis. Infect Immun. 2000;68(3):1125-33.
501. YU XJ, ZHANG XF, MCBRIDE JW, ZHANG Y, WALKER DH. Phylogenetic relationships of *Anaplasma marginale* and '*Ehrlichia platys*' to other *Ehrlichia* species determined by GroEL amino acid sequences. Int J Syst Evol Microbiol. 2001;51(Pt3):1143-6.
502. ZEIDNER NS, BURKOT TR, MASSUNG R, NICHOLSON WL, DOLAN MC, RUTHERFORD JS, BIGGERSTAFF BJ, MAUPIN GO. Transmission of the agent of human granulocytic ehrlichiosis by *Ixodes spinipalpis* ticks: evidence of an enzootic cycle of dual infection with *Borrelia burgdorferi* in Northern Colorado. J Infect Dis. 2000;182(2):616-9.
503. ZEMAN P, CINATL J, KABICKOVA H. Sodium valproate facilitates the propagation of granulocytic ehrlichiae (*Anaplasma phagocytophilum*) in HL-60 cells. Exp Appl Acarol. 2002a;28(1-4):203-8.
504. ZEMAN P, PAZDIORA P, CINATL J. HGE antibodies in sera of patients with TBE in the Czech Republic. Int J Med Microbiol. 2002b;291(Suppl33):190-3.
505. ZEMAN P, BENE C. A tick-borne encephalitis ceiling in Central Europe has moved upwards during the last 30 years: possible impact of global warming? Int J Med Microbiol. 2004;293(Suppl37):48-54.
506. ZHANG Y, OHASHI N, LEE EH, TAMURA A, RIKIHISA Y. *Ehrlichia sennetsu* groE operon and antigenic properties of the GroEL homolog. FEMS Immunol Med Microbiol. 1997;18(1):39-46.
507. ZHANG JZ, POPOV VL, GAO S, WALKER DH, YU XJ. The developmental cycle of *Ehrlichia chaffeensis* in vertebrate cells. Cell Microbiol. 2007;9(3):610-8.
508. ZHI N, OHASHI N, RIKIHISA Y, HOROWITZ HW, WORMSER GP, HECHEMY K. Cloning and expression of the 44-kilodalton major outer membrane protein gene of the human granulocytic ehrlichiosis agent and application of the recombinant protein to serodiagnosis. J Clin Microbiol. 1998;36(6):1666-73.
509. ZHI N, OHASHI N, TAJIMA T, MOTT J, STICH RW, GROVER D, TELFORD SR 3RD, LIN Q, RIKIHISA Y. [Transcript heterogeneity of the p44 multigene family in a human granulocytic ehrlichiosis agent transmitted by ticks]. Infect Immun. 2002;70(3):1175–84. [Erratum in: Infect Immun 2002;70(8):4754].

# APPENDIXES

# APPENDIX 1. CARRAÇAS ASSOCIADAS A PATOLOGIAS INFECCIOSAS EM PORTUGAL

SILVA MM, SANTOS AS, FORMOSINHO P, BACELLAR F

REVISION ARTICLE

ACTA MÉDICA PORTUGUESA 2005; 19:39-48

ARTIGO REVISÃO

Acta Med Port 2006; 19: 39-48

# CARRAÇAS ASSOCIADAS A PATOLOGIAS INFECCIOSAS EM PORTUGAL

M.ª MARGARIDA SILVA, A SOFIA SANTOS, PERPÉTUA FORMOSINHO, FÁTIMA BACELLAR
Centro de Estudos de Vectores e Doenças Infecciosas, Instituto Nacional de Saúde Dr. Ricardo Jorge. Lisboa.

**RESUMO**

Os ixodoideos, também designados por carraças, são artrópodes hematófagos estritos ectoparasitas de vertebrados terrestres. Estes artrópodes estão presentes em quase todas as regiões zoogeográficas. Embora sejam considerados zoofílicos, são várias as espécies associadas à transmissão ao Homem de importantes agentes etiológicos, responsáveis pelo aparecimento de diversas doenças infecciosas. Julgamos por isso oportuno, contribuir desta forma para uma revisão dos aspectos relevantes, relacionados com a caracterização destes artrópodes, como vectores de agentes infecciosos em Portugal, abordando aspectos biológicos, ecológicos e epidemiológicos com interesse em Saúde Pública.

*Palavras-Chave – Ixodídeos; Agentes transmitidos por ixodídeos; Saúde Pública; Portugal*

**SUMMARY**

**TICKS ASSOCIATED TO INFECTIOUS PATHOLOGIES IN PORTUGAL**

Ticks are hematophagos arthropods that parasitize terrestrial vertebrates. They are world wide, living in almost all terrestrial regions. Although mainly associated to animals, there are several tick species that bite humans and transmit tick-borne agents causing important infectious disease. In this paper the authors revise the most outstanding aspects of those arthropods as vectors of infectious pathogens in Portugal, focusing biologic, ecologic and epidemiologic features with Public Health interest.

*Key-Words – Ticks; Tick-borne pathogens; Public Health; Portugal*

*Recebido para publicação: 8 de Março de 2005*

## INTRODUÇÃO

Os ixodoideos, vulgarmente designados por carraças, são artrópodes ectoparasitas hematófagos estritos. Existem em quase todas as regiões zoogeográficas, parasitando uma ampla variedade de hospedeiros como mamíferos, aves, répteis e anfíbios. São conhecidas aproximadamente 850 espécies distribuídas por três famílias: Nuttallielidae, Argasidae e Ixodidae. *Nuttalliela namaqua*, é o único representante da família Nuttallielidae. É uma espécie rara, apenas conhecida na África do Sul, que possui características intermédias entre os elementos das outras famílias e cujo papel na transmissão de agentes infecciosos é desconhecido[1,2]. Na família Argasidae são conhecidas cerca de 170 espécies que, pela ausência de escudo dorsal, são designadas argasídeos ou "carraças de corpo mole". Da família Ixodidae fazem parte cerca de 650 espécies de ixodídeos, vulgarmente designados por "carraças de corpo duro" pela presença de escudo dorsal. Nestas duas últimas famílias, cerca de 10% das espécies conhecidas estão associadas à transmissão ao Homem e a outros vertebrados, de agentes patogénicos responsáveis por várias doenças infecciosas como ricketsioses, borrelioses, ehrlichioses, tularémia, arboviroses, babesioses, entre outras patologias[1]. Contudo, é a família Ixodidae a que se reveste de maior importância médica pelo número de espécies implicadas na transmissão de agentes patogénicos, facto que nos levou a considerar uma revisão sobre este tema.

### Ixodídeos

O ciclo biológico dos ixodídeos compreende quatro fases evolutivas: uma fase inactiva - ovo e três fases activas - larva, ninfa e adulto (macho ou fêmea) (Figura 1). Durante as fases activas, os ixodídeos alternam entre períodos de intensa actividade (procura de hospedeiro e alimentação) e períodos não activos (metamorfose e diapausa), necessitando sempre de uma refeição de sangue para passarem ao estado evolutivo seguinte. Ao notável sucesso destes artrópodes, como vectores potenciais de agentes patogénicos, são associadas características biológicas singulares[2,3] (Quadro I), que os colocam, imediatamente a seguir aos mosquitos, como os artrópodes vectores de maior importância em Saúde Pública[1,2].

### Vectores mecânicos / Vectores biológicos

Na transmissão efectiva do agente infeccioso os ixodídeos podem actuar quer como vectores mecânicos, quer como vectores biológicos[4]. No primeiro caso, a sobrevivência do agente depende da sua capacidade para suportar as condições do tracto digestivo do artrópode, até ser transmitido ao hospedeiro vertebrado. O agente patogénico não se multiplica no vector e o artrópode apenas o transmite mecanicamente, de um hospedeiro vertebrado para outro. No caso em que o ixodídeo é vector biológico, o agente infeccioso invade o


*Fig 1 - Fases evolutivas do ciclo de vida dos ixodídeos. Legenda: 1-ovo; 2-larva; 3-ninfa; 4-macho; 5-fêmea.*

*Quadro I - Características biológicas mais importantes dos ixodídeos (adaptado de Sonenshine, 1991)*

| | |
|---|---|
| IXODÍDEOS | Hematofagia obrigatória (larvas, ninfas e adultos) |
| | Apresentam ciclos de vida mono, di e trifásicos, consoante necessitam de se alimentar em um, dois ou três hospedeiros vertebrados[a] |
| | A alimentação é um processo geralmente lento o que propicia um alargado período de interacção com o hospedeiro |
| | Todas as fases de desenvolvimento ingerem grandes volumes de sangue |
| | Digestão gradual e intracelular, que ocorre sem a presença de enzimas no lúmen intestinal |
| | Existência de transmissão transtadial[b] ou transmissão horizontal |
| | Existência de transmissão transovárica[c] ou transmissão vertical |
| | Existência de um sistema sensorial desenvolvido[d] |
| | Diapausa[e] |
| | Grande longevidade e elevadas taxas de prolificidade |
| | Distribuição por quase todos os habitats terrestres e reduzido número de predadores naturais |

[a] No caso do ciclo monofásico todas as fases de desenvolvimento do artrópodo alimentam-se no mesmo hospedeiro, ao contrário do ciclo difásico, em que apenas as fases imaturas partilham o mesmo hospedeiro. Contudo, mais de 90% das espécies apresentam ciclos trifásicos (em que cada fase de desenvolvimento se alimenta num hospedeiro diferente), o que aumenta a probabilidade de serem infectadas e a capacidade de transmitir a infecção.
[b] As metamorfoses não envolvem a degeneração e regeneração total de cada órgão, pelo que, os microrganismos sobrevivem à muda de fase evolutiva, processo denominado por transmissão transtadial ou transmissão horizontal.
[c] Em algumas associações ixodídeo/agente infeccioso ocorre a invasão do sistema reprodutor, permitindo assim a transmissão da infecção à progenitura, processo denominado por transmissão transovárica. Neste caso os ixodídeos são considerados também, para além de vectores, reservatórios naturais do agente.
[d] Um sistema sensorial bem desenvolvido permite aos ixodídeos detectar a presença de potenciais hospedeiros (quer pelas suas emissões de dióxido de carbono, ácido láctico, amoníaco e outros odores, como pelas vibrações que causam). Deste modo, podem ocupar posições estratégicas, em trilhos de passagem, que aumentam as probabilidades de sucesso no encontro com novos hospedeiros.
[e] Adaptação que lhes permite a sobrevivência em ambientes desfavoráveis.

corpo do artrópode, proliferando nos seus tecidos antes de ser transmitido a um outro hospedeiro vertebrado. Esta situação é a mais frequente, verificando-se para a maioria dos agentes associados a estes artrópodes[4,5].

**Capacidade vectorial / Competência dos vectores**

Para a avaliação da eficácia de um ixodídeo como vector existem dois parâmetros principais: a capacidade vectorial e a competência do vector. A capacidade vectorial é a habilidade que uma determinada espécie tem, no tempo e no espaço, para transmitir o agente patogénico. Factores como a dimensão da população dos artrópodes, a longevidade, o número de posturas e o próprio comportamento alimentar afectam a capacidade vectorial de uma determinada população. A competência de um vector é a capacidade intrínseca que um ixodídeo tem para manter a infecção e consequentemente transmitir biologicamente o agente infeccioso, durante a alimentação. A Organização Mundial de Saúde, em 1985, definiu "vector competente" como aquele que possui um limiar de infecção baixo, que apresenta um reduzido período de incubação extrínseca[a)] e uma elevada eficácia de transmissão[6]. Assim, para que um ixodídeo possa ser considerado um vector competente de determinado agente infeccioso é necessário que apresente evidências, não só da sua manutenção, mas também da capacidade de o transmitir a um hospedeiro susceptível.

**Infecção/transmissão do agente infeccioso**

A infecção de um ixodídeo com um agente infeccioso pode ocorrer através de vários processos, nomeadamente por: I. transmissão transtadial e/ou transovárica; II. alimentação num hospedeiro vertebrado infectado; III. *cofeeding*[b)].

De um modo geral, a transmissão do agente infeccioso a um hospedeiro vertebrado deve-se à picada de um ixodídeo infectado, com a consequente inoculação de secreções salivares contendo o agente. Porém, e dependendo da natureza do agente infeccioso, a transmissão também pode ocorrer, nas seguintes situações: I. Quando o ixodídeo se alimenta, estando as suas peças bucais contaminadas com sangue infectado, proveniente de um hospedeiro ao qual se fixou anteriormente, mas no qual não completou a refeição; II. Quando o ixodídeo infectado liberta fezes contaminadas, sobre a descontinuidade cutânea, resultante do acto alimentar ou quando ocorre o seu esmagamento sobre esse local; III. Quando o ixodídeo infectado é deglutido[4,5].

Embora os ixodídeos sejam zoofílicos, têm sido observadas, com alguma frequência, diferentes espécies a parasitar o Homem, e que têm sido implicadas na transmissão de diversos agentes patogénicos (Quadro II). Esta situação não é atribuída a uma tendência antropofílica, mas sim à oportunidade de contacto com o Homem, o qual se torna um hospedeiro acidental.

***Quadro II** – Principais agentes patogénicos transmitidos ao Homem por ixodídeos.*

| Agentes patogénicos | Doença | Distribuição geográfica | Principais vectores |
|---|---|---|---|
| **Vírus** | | | |
| Encefalite transmitida por carraça (TBE) | Meningoencefalite | Europa, Ásia | *Ixodes ricinus*, *I. persulcatus* |
| Febre hemorrágica Crimeia-Congo (CCHF) | Febre hemorrágica | Europa, Ásia, África | *Hyalomma marginatum marginatum, H. m. rufipes, Haemaphysalis spp.* |
| Febre da carraça do Colorado (CTF) | Doença febril sistémica | América do Norte | *Dermacentor andersoni* |
| **Bactérias** | | | |
| *Rickettsias* | | | |
| *Rickettsia rickettsii* | Febre exantemática das Montanhas Rochosas | América do Norte<br>Central e do Sul | *Dermacentor spp.*<br>*Amblyomma spp., Rhipicephalus spp.* |
| *R. conorii* | Febre botonosa ou escaro-nodular | Sub-região Mediterrânica<br>Ásia e Africa | *Rhipicephalus spp.*,<br>*Haemaphysalis spp., Amblyomma spp.*,<br>*Hyalomma spp.* |
| *R. japonica* | Febre exantemática oriental | Japão | *Haemaphysalis spp.* |
| *R. africae* | Febre da carraça africana | África | *Amblyomma hebraeum* |
| *R. slovaca* | Tibola (Linfadenopatia causada pela picada da carraça) | Europa | *Dermacentor marginatus* |
| *R. helvetica* | Perimiocardite crónica | Europa | *Ixodes ricinus* |
| *Ehrlichias* | | | |
| *Ehrlichia chaffeensis* | Ehrlichiose monocítica humana | EUA | *Amblyomma americanum* |
| *Anaplasma phagocytophilum* | Ehrlichiose granulocítica humana (HGE) | EUA<br>Europa | *Ixodes scapularis*<br>*I. ricinus;* |
| Borrelias | | | |
| *Borrelia burgdorferi sl.* | Borreliose de Lyme | EUA<br>Europa<br>África<br>Austrália | *Ixodes scapularis, I. pacificus*<br>*I. ricinus, I. persulcatus*<br>*I. persulcatus*<br>*I. holocyclus* |
| Outras bactérias | | | |
| *Francisella tularensis* | Tuarémia | Europa, Ásia, Africa, América do Norte | *Haemaphysalis leporipalustris, Dermacentor marginatus, D. variabilis, D. andersoni, D. americanum* |
| *Coxiella burnetii* | Febre Q | Europa, Ásia, África, América do Norte | *Rhipicephalus spp.* |
| **Protozoários** | | | |
| Babesias | | | |
| *Babesia microti* | Babesiose humana | EUA | *I. scapularis* |
| *B. divergens* | Babesiose humana | Europa | *I. ricinus* |

Os agentes patogénicos podem ser encontrados em focos naturais, que envolvem diversas espécies roedores e outros mamíferos silváticos. A infecção humana resulta do contacto do Homem com esses ambientes, através de actividades relacionadas com a profissão (agricultura, pastorícia, etc.) ou de lazer (campismo, caça, passeios pedestres, etc.). Contudo, alguns agentes podem ainda estabelecer focos secundários rurais ou urbanos (como é o caso das rickettsias), em que diversos mamíferos domésticos e roedores, que vivem na dependência humana, desempenham um papel importante. Neste caso, pode ocorrer infecção sem contacto do Homem com os focos naturais[5].

No Homem, a infecção resulta na maioria dos casos da picada do ixodídeo infectado. Considera-se no entanto, que é necessário existir um determinado período de fixação do artrópode para haver uma transmissão efectiva do agente infeccioso ao homem. Por exemplo, no caso de agentes rickettsiales pode ser de 6-20h[7]. Na maioria dos casos, qualquer uma das formas evolutivas dos ixodídeos é capaz de transmitir o agente infeccioso, sendo particularmente importantes as fases imaturas que, pelas suas reduzidas dimensões, são dificilmente detectadas. É, sobretudo, ao estado de ninfa que se deve grande parte das infecções no Homem. Para além da picada, também está descrita na literatura a infecção por contaminação de descontinuidades cutâneas ou de mucosas, quer com fluídos corporais dos artrópodes infectados, por exemplo quando se procede à desparasitação de animais, quer com as fezes dos ixodídeos (esta última situação ocorre concretamente com artrópodes infectados por *Coxiella sp.* ou *Francisella sp.*)[3]. Convém ainda referir que, para alguns agentes, a infecção humana não resulta exclusivamente do contacto com os ixodídeos. A infecção com o vírus TBE (Tick-borne encephalitis), pode ser adquirida pela ingestão de produtos derivados de animais infectados, nomeadamente leite[8]. Adicionalmente, *Coxiella sp.* e *Francisella sp.* são espécies muito resistentes às condições ambientais, podendo contaminar os solos e reservas de água. Deste modo, a infecção humana pode resultar do contacto com estes ambientes e/ou com fluídos biológicos contaminados, provenientes da manipulação de animais infectados[3].

**Principais espécies ixodológicas com interesse médico**

Portugal apresenta condições climáticas, ecológicas e ambientais favoráveis ao desenvolvimento de várias espécies ixodológicas. Actualmente, estão identificadas 21 espécies classificadas na família Ixodidae, das quais várias são reconhecidos vectores de agentes etiológicos causadores de doença no Homem (Quadro III). Pela importância que alguns destes *taxa* representam, em termos de Saúde Pública, realçaremos algumas das espécies de ixodídeos que apresentam um papel, real ou potencial, na transmissão de agentes infecciosos ao Homem (Quadro IV, Figura 2).

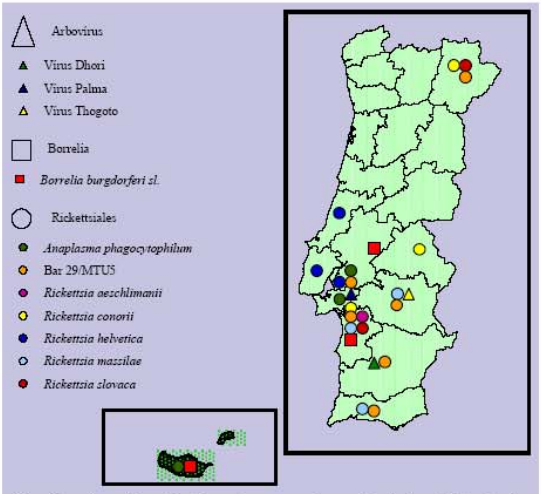



***Fig. 2** – Localização dos microrganismos isolados /detectados em ixodídeos*

***Quadro III** - Sistemática das espécies de Ixodidae existentes em Portugal e seus hospedeiros preferenciais.*

| Família *Ixodidae* | | | | |
|---|---|---|---|---|
| Género | | | | |
| *Rhipicephalus* | *Ixodes* | *Dermacentor* | *Hyalomma* | *Haemaphysalis* |
| Espécie / Hospedeiro preferêncial | | | | |
| *R.sanguineus* / Canídeos domésticos | *I. ricinus* / Mamíferos silváticos | *D. marginatus* / Bovinos | | |
| *R. turanicus* / Ovinos | *I. hexagonus* / Mamíferos silváticos | *D. reticulatus*[2] / Canídeos domésticos e silváticos | *H. lusitanicum* / Bovinos | *H. punctata* / Mamíferos domésticos e silváticos |
| *R. pusillus* / Pequenos mamíferos silváticos | *I. vespertilionis* / Quirópteros | | *H. marginatum* / Bovinos | *H. inermis* / Mamíferos silváticos |
| *R. bursa* / Caprinos | *I. ventalloi* / Pequenos mamíferos silváticos | | | *H. hispanica* / Pequenos mamíferos silváticos |
| *R. annulatus*[1] / Bovinos | *I. bivari* / Pequenos mamíferos silváticos | | | |
| | *I. canisuga* / Mamíferos silváticos | | | |
| | *I. simplex* / Quirópteros | | | |
| | *I. acuminatus* / Pequenos mamíferos silváticos | | | |
| | *I. frontalis* / Aves silváticas | | | |

[1] *Espécie também denominada por Boophilus annulatus;* [2] *Espécie anteriormente denominada por Dermacentor pictus*

*Quadro IV – Microrganismos detectados/ isolados de ixodídeos em Portugal.*

| Espécie ixodológica (Vector/Reservatório) | Agente Etiológico | Patologia associada | Patogenia em Portugal | Assinalado em Portugal |
|---|---|---|---|---|
| *Dermacentor marginatus* | *Rickettsia slovaca* | Tibola | Desconhecida | Bacellar *et al*, 1995 |
| | *Borrelia burgdorferi sl* | Borreliose de Lyme | Conhecida | Baptista *et al*, 2004 |
| *Haemaphysalis punctata* | Vírus Palma | _ | Desconhecida | Filipe *et al*, 1994 |
| *Hyalomma marginatum* | *Rickettsia aeschlimanii* | _ | Desconhecida | Bacellar *et al*, 1999 |
| | Vírus Dhori | _ | Desconhecida | Filipe & Casals, 1979 |
| | *Borrelia burgdorferi sl* | Borreliose de Lyme | Conhecida | Baptista *et al*, 2004 |
| *Ixodes ricinus* | *Anaplasma phagocytophilum* | Ehrlichiose granulocítica humana | Desconhecida | Santos *et al*, 2004a |
| | *Borrelia burgdorferi sl.* | Borreliose de Lyme | Conhecida | Núncio *et al*, 1993 |
| | *Rickettsia helvetica* | Perimiocardite crónica | Desconhecida | Bacellar *et al*, 1999 |
| *Ixodes ventalloi* | *Anaplasma phagocytophilum* | Ehrlichiose granulocítica humana | Desconhecida | Santos *et al*, 2004a |
| | *Rickettsia helvetica* | Perimiocardite crónica | Desconhecida | Bacellar *et al*, 1999 |
| *Rhipicephalus sanguineus* | *Rickettsia conorii* | Febre botonosa ou escaro-nodular | Conhecida | Bacellar *et al*, 1995 |
| | *R. massiliae* | _ | Desconhecida | Bacellar *et al*, 1995 |
| | Vírus Thogoto | __ | Desconhecida | Filipe & Calisher, 1984 |
| *Rhipicephalus turanicus* | *Rickettsia massiliae* | _ | Desconhecida | Bacellar *et al*, 1995 |
| | Bar 29/MTU5 | _ | Desconhecida | Bacellar *et al*, 1995 |

**Género *Rhipicephalus***

Neste género encontra-se classificada uma das espécies de maior importância no nosso País, *Rhipicephalus sanguineus*. Esta é uma espécie que existe em quase todas as regiões do mundo, com a excepção das zonas circumpolares. Este ixodídeo encontra-se distribuído de Norte a Sul do País estando, do ponto de vista ecológico, adaptado a uma grande variedade de climas e hospedeiros vertebrados. Parasita numerosos animais silváticos e todas as espécies de animais domésticos, estando particularmente associada ao cão doméstico - *Canis familiaris*[9,10]. Do ponto de vista biológico, *R. sanguineus* tem uma evolução do tipo trifásico[c)] e ditrópico[d)], sendo as formas adultas exofílicas[e)] e as imaturas endofílicas, evoluindo habitualmente nos locais onde os hospedeiros se refugiam.

Porém, no caso em que as populações de ixodídeos vivem em estrita dependência do seu hospedeiro preferencial (o cão doméstico), o ciclo alimentar manifesta-se essencialmente de forma monotrópica e endofílica, alojando-se tanto as formas imaturas com os adultos nos canis ou dentro das próprias habitações do Homem. As maiores densidades populacionais foram encontradas nos meses mais quentes (Julho e Agosto), pelo que, esta espécie, está melhor adaptada a temperaturas altas, não sendo exigente quanto a humidade relativa, sobrevivendo com facilidade em climas secos. As formas adultas são encontradas em quase todos os meses do ano, com um incremento na altura de Primavera/Verão, resultante de uma maior actividade e abundância do vector. A maior actividade das formas imaturas está sobretudo concentrada nos meses de verão. Quando as condições ambientais (temperatura, humidade relativa, fotoperíodo) são favoráveis esta espécie pode completar anualmente 2 ou 3 ciclos de vida, com posturas na ordem dos 5000 ovos.

A *R. sanguineus* cabe-lhe a transmissão de estirpes do complexo-*Rickettsia conorii*, agentes da febre botonosa ou escaro-nodular[11,12] que, no nosso País, é a principal doença associada a ixodídeos. Classificada como uma doença de declaração obrigatória, apresenta uma taxa de incidência de $9.8/10^5$ habitantes sendo uma das mais elevadas face aos países da bacia do Mediterrâneo[13]. A febre botonosa apresenta um período de incubação de aproximadamente uma semana, muitas vezes assintomático, após o qual surge um quadro clínico de início súbito caracterizado por cefaleias, mialgias, artralgias e prostração acentuadas. Podem ainda ocorrer sintomas gastrintestinais, tais como vómitos e diarreia. Após uma semana, aparecem manchas na pele - o exantema maculo–papular, que atinge as palmas das mãos e as plantas dos pés, podendo poupar a face. No local da picada da carraça forma-se uma escara, lesão com crosta negra de 0,5–2 cm de diâmetro, com halo eritematoso. Contudo, pode ter um aspecto pustuloso ou ser apenas uma pápula eritematosa de pequeno diâmetro[14].

De *R. sanguineus* foram ainda isoladas outras rickettsias, consideradas não patogénicas como *R. massiliae* (também isolada de *R. turanicus*) e um agente ainda sem nomenclatura oficial, Bar 29/MTU5[11,16]. Este ixodídeo está também associado a *Coxiella burnetti* (agente etiológico da febre Q). Embora este último agente infeccioso circule em território nacional e já tenha sido por nós detectado em *Rhipicephalus spp.* (assim como em *Haemaphysalis spp.*) por técnicas de biologia molecular,

ainda não foi possível o seu isolamento. A febre Q é uma doença polimorfa, quando aguda pode apresentar-se como uma pneumonia ou um síndroma gripal ou pode tornar-se crónica e manifestar-se por endocardite, infecção valvular, osteomielite e hepatite crónica. Geralmente, *C. burnetti* é transmitida ao Homem por aerossóis de produtos animais infectados e não pela picada do artrópode, não estando excluída a hipótese das suas fezes manterem o agente viável e ser uma fonte de infecção.

A partir de *R. sanguineus* foi também possível o isolamento de agentes virais nomeadamente o vírus Thogoto[15], cuja responsabilidade na patologia de doenças infecciosas ainda não está esclarecida (Quadro IV).

**Género *Ixodes***

No género *Ixodes*, é a espécie *Ixodes ricinus* que se reveste de maior importância médica. Este ixodídeo apresenta uma grande área de expansão, ocupando toda a Europa, bem como a África Mediterrânica e a Ásia Menor. Em Portugal, embora com uma distribuição desigual, pode ser encontrado de Norte a Sul, predominantemente nas regiões e/ou locais que apresentem uma cobertura vegetal considerável e onde se verifiquem elevados níveis de humidade relativa (acima de 90%). É uma espécie muito dependente do estado higrométrico do ar e da temperatura, cujo equilíbrio lhe é essencial. A insuficiência de cobertura vegetal e as temperaturas elevadas comprometem a vitalidade das formas evolutivas, levando-as a procurar refúgios apropriados à sua sobrevivência e a utilizar, frequentemente, mecanismos de defesa como a diapausa. Esta espécie apresenta uma excepcional capacidade de adaptação a diversos hospedeiros, parasitando tanto mamíferos domésticos como silváticos, aves e alguns lacertídeos, sendo também frequentemente detectada a parasitar o Homem[9,10]. Do ponto de vista biológico é uma espécie de evolução trifásica, exofílica e politrópica. A sua actividade estende-se por todo o ano, porém com alguma sazonalidade. As formas adultas estão activas durante os períodos menos quentes do ano (Setembro/Março) interrompendo a actividade durante o período de Verão. Ao contrário, as formas imaturas têm maior actividade nos meses de Primavera/Verão (Abril/Junho), justificando a sazonalidade observada nas doenças associadas a este artrópode. Na Natureza *I. ricinus* apresenta um ciclo de vida anual, podendo estender-se até três anos, com posturas não superiores a 3000 ovos.

*I. ricinus* é o principal vector de *Borrelia burgdorferi sl*, agente etiológico da borreliose de Lyme. Esta patologia é considerada multissistémica e multifásica, com sintomatologia variável, podendo ser enquadrada segundo o predomínio das manifestações em: síndromas febris, sintomatologia predominantemente dermatológica, osteo-articular e neurológica, atingindo preferencialmente a pele, as articulações, o sistema nervoso e o coração. Numa fase precoce da doença, o diagnóstico clínico baseia-se na presença do *eritema migrans* (lesão dermatológica característica) que, quando presente, é suficiente para estabelecer o diagnóstico clínico. Até ao momento, a borreliose de Lyme é a única doença comprovadamente associada a *I. ricinus* no nosso país, porém, esta espécie poderá ter um papel relevante na transmissão de outros agentes infecciosos. Deste ixodídeo têm sido isoladas estirpes de *Rickettsia helvetica*[11,16] agente que em outras regiões da Europa foi associado a periomiocardite crónica e à morte

***Quadro IV** – Microrganismos detectados/ isolados de ixodídeos em Portugal*

| Espécie ixodológica (Vector/Reservatório) | Agente Etiológico | Patologia associada | Patogenia em Portugal | Assinalado em Portugal |
|---|---|---|---|---|
| *Dermacentor marginatus* | *Rickettsia slovaca* | Tibola | Desconhecida | Bacellar *et al*, 1995 |
| | *Borrelia burgdorferi sl* | Borreliose de Lyme | Conhecida | Baptista *et al*, 2004 |
| *Haemaphysalis punctata* | Vírus Palma | _ | Desconhecida | Filipe *et al*, 1994 |
| *Hyalomma marginatum* | *Rickettsia aeschlimanii* | _ | Desconhecida | Bacellar *et al*, 1999 |
| | Vírus Dhori | _ | Desconhecida | Filipe & Casals, 1979 |
| | *Borrelia burgdorferi sl* | Borreliose de Lyme | Conhecida | Baptista *et al*, 2004 |
| *Ixodes ricinus* | *Anaplasma phagocytophilum* | Ehrlichiose granulocítica humana | Desconhecida | Santos *et al*, 2004a |
| | *Borrelia burgdorferi sl.* | Borreliose de Lyme | Conhecida | Núncio *et al*, 1993 |
| | *Rickettsia helvetica* | Perimiocardite crónica | Desconhecida | Bacellar *et al*, 1999 |
| *Ixodes ventalloi* | *Anaplasma phagocytophilum* | Ehrlichiose granulocítica humana | Desconhecida | Santos *et al*, 2004a |
| | *Rickettsia helvetica* | Perimiocardite crónica | Desconhecida | Bacellar *et al*, 1999 |
| *Rhipicephalus sanguineus* | *Rickettsia conorii* | Febre botonosa ou escaro-nodular | Conhecida | Bacellar *et al*, 1995 |
| | *R. massiliae* | _ | Desconhecida | Bacellar *et al*, 1995 |
| | Vírus Thogoto | __ | Desconhecida | Filipe & Calisher, 1984 |
| *Rhipicephalus turanicus* | *Rickettsia massiliae* | _ | Desconhecida | Bacellar *et al*, 1995 |
| | Bar 29/MTU5 | _ | Desconhecida | Bacellar *et al*, 1995 |

de jovens adultos[17].

Estudos recentes, revelaram ainda a presença nesta espécie de *Anaplasma phagocytophilum*[f)] (agente também detectado em *I. ventalloi*)[19]. Este agente é responsável por uma zoonose designada anaplasmose granulocítica humana (actual designação para ehrlichiose granulocitica humana), que ocorre principalmente nos Estados Unidos mas que também já foi assinalada em algumas regiões da Europa[20]. Esta doença costuma cursar com quadros febris moderados, geralmente de prognóstico benigno, acompanhados de uma sintomatologia inespecífica como febre, arrepios, mal-estar geral, cefaleias e mialgias, entre outros, e alterações hematológicas como trombocitopénia, leucopénia e aumento dos valores das transaminases[21]. Convém ainda salientar o facto de *I. ricinus* estar associado à virose provocada pela actividade do vírus TBE que é sem dúvida a arbovirose mais importante que afecta o Homem na Europa[22]. A maioria das infecções por este vírus são assintomáticas, contudo podem evoluir para uma infecção do sistema nervoso central. Neste caso, a doença apresenta um curso bifásico, em que a primeira fase é caracterizada por febre, cefaleias, náuseas, vómitos e uma segunda fase com sinais e sintomas de meningite e meningoencefalite. Este ixodídeo está também implicado na transmissão de protozoários do género *Babesia*, que noutros países europeus tem sido implicado em casos de babesiose humana. Muito embora este agente ainda não tenha sido detectado no nosso país a sua presença em Espanha[23] deixa em aberto a possibilidade da sua ocorrência no território nacional.

**Género *Dermacentor***

Neste género, *Dermacentor marginatus* é a espécie que mais se destaca, quer pela frequência com que é detectada, quer pelo variado número de hospedeiros que parasita, abrangendo, praticamente, todos os mamíferos domésticos, uma variada gama de animais silváticos e o Homem[9,10]. Esta espécie encontra-se presente na Europa, Ásia Central e no Norte de África, estando em Portugal distribuída por todo o País.

Ocorre em regiões de clima temperado e seco, no entanto suporta com facilidade temperaturas mais elevadas, não sendo também muito exigente em humidade.

Do ponto de vista biológico, *D. marginatum* é uma espécie de ciclo trifásico e ditrópico, com as formas adultas exofílicas e as fases imaturas endofílicas. As formas adultas apresentam uma maior actividade na altura do Outono/Inverno e os estados imaturos de larva e ninfa na Primavera/Verão. No entanto, poder-se-ão encontrar todas as fases de desenvolvimento em qualquer época do ano. Na Natureza esta espécie realiza um ciclo de vida por ano, com posturas que podem chegar até aos 7000 ovos.

Embora com menor relevância que as espécies anteriormente referidas *Dermacentor marginatus* também tem sido associada à parasitação do Homem. A partir desta espécie, foi isolada *Rickettsia slovaca*, agente que no nosso país apresentou uma elevada prevalência[16,24]. *R. slovaca*, tida como uma rickettsia não patogénica, é hoje associada a uma nova patologia denominada TIBOLA (tick-borne lymphadenopathy ou linfoadenopatia causada pela picada de carraça)[25]. As manifestações clínicas são descritas como uma reacção no local da picada, geralmente localizada na cabeça, em pápula ou vesícula que evolui para uma lesão exsudativa, necrótica, formando-se uma crosta e alopécia, acompanhada de adenopatias cervicais e submaxilares. Um dos sintomas, febre baixa, pode persistir por meses e a alopécia por anos[25]. Importa no entanto referir que até ao momento não foram descritos casos de TIBOLA em Portugal.

**Género *Hyalomma***

Em Portugal é sem dúvida a espécie *Hyalomma marginatum* a que assume um papel preponderante dentro deste género. Esta espécie apresenta uma larga distribuição geográfica, sendo muito comum na Ásia, em algumas regiões de África e nos países da orla do mediterrânio. Este ixodídeo ocorre de Norte a Sul do País e do ponto de vista biológico é considerado uma espécie de ciclo quer trifásico quer bifásico, endofílica ou exofílica, monotrópica ou ditrópica, consoante o tipo de hospedeiros que parasita. As fases adultas são encontradas, frequentemente, a parasitar animais domésticos, nomeadamente, bovinos[9,10]. As formas imaturas, larvas e ninfas, têm sido encontradas a parasitar aves[26], podendo também ocorrer em pequenos animais silváticos como ouriços-caixeiros e coelhos[9,10]. Em Portugal, o Homem também surge como hospedeiro desta espécie, sendo este trabalho o primeiro registo desta ocorrência. Em termos climáticos esta espécie está adaptada a climas quentes e secos, não sendo por isso muito exigente do ponto de vista higrométrico. O período de maior actividade das formas adultas é durante a Primavera–Verão, enquanto as formas imaturas de larvas e ninfas são encontradas, com maior facilidade, no final do Verão e durante o período do Outono-Inverno. Na Natureza, *H. marginatum* pode realizar mais de um ciclo de vida anual, com posturas na ordem dos 11.000 ovos.

*H. marginatum* está associado ao isolamento da *Rickettsia aeschilimanii*[27] cuja intervenção na patologia das doenças infecciosas ainda não está totalmente averiguada em Portugal. Em França, estão descritos al-

guns casos clínicos associados a esta rickettsia, acompanhados de febre, escara, exantema cutâneo e linfangite. Todos os casos ocorreram na Primavera e estão relacionados com a migração de aves que introduziram o vector infectado[28]. Nesta espécie regista-se também o isolamento do vírus Dhori, cuja responsabilidade em patologias humanas não está ainda esclarecida[29]. Em outros Países, nomeadamente na ex-União Soviética, Mauritânia, Senegal, Egipto e Grécia, *H. marginatum* está associado à transmissão de outros vírus, nomeadamente o vírus da febre hemorrágica Crimeia-Congo.

**Género *Haemaphysalis***

*Haemaphysalis punctata* é a espécie que se destaca dentro deste género. Embora não haja registos, no país, da sua presença no Homem, foi já encontrada numa variada gama de animais domésticos, ocorrendo também em animais silváticos e em aves[9,10]. A área de distribuição desta espécie, engloba toda a Europa e os países africanos da orla Mediterrânica, nomeadamente Egipto, Líbia, Tunísia, Argélia e Marrocos. Em Portugal encontra–se distribuída de Norte a Sul. Do ponto de vista biológico, *H. punctacta* é uma espécie de ciclo trifásico, exofílica e politrópica. As formas adultas tem maior actividade no período de Outono-Inverno, ao contrário dos estados imaturos de larva e ninfa cujo período de maior actividade é a Primavera-Verão. Prefere zonas de clima temperado a frio, não sendo muito exigente nas condições de humidade relativa. Na Natureza esta espécie apresenta um ciclo de vida anual, com posturas na ordem dos 3000 ovos.

A partir de *H. punctacta* foi possível isolar um agente serologicamente caracterizado como um novo vírus, pertencente ao grupo antigénico Bhanja, denominado por vírus Palma[30]. Até ao momento, não está esclarecido qual o papel que este vírus pode desempenhar em Saúde Pública. Sabe-se, porém, que outras espécies de ixodídeos, nomeadamente, *D. marginatus* e *R. sanguineus* podem também permitir laboratorialmente a sobrevivência deste vírus[31].

## CONSIDERAÇÕES FINAIS

A integração dos estudos de sistemática e ecobiologia dos ixodídeos, a par da identificação de microrganismos patogénicos de que são vectores e reservatórios naturais, é a grande aposta que vem sendo desenvolvida há quase duas décadas pela equipa do Centro de Estudos de Vectores e Doenças infecciosas, Instituto Nacional de Saúde Dr. Ricardo Jorge. Estes esforços concertados são indispensáveis para o desenvolvimento de programas de planeamento e controlo dos ixodídeos e das doenças por estes vectoriadas, possibilitando o delineamento de zonas de potencial risco para o Homem.

## AGRADECIMENTOS

Os autores gostariam de expressar o seu agradecimento a José Poças (Director do Serviço de Infecciologia do Hospital de São Bernardo) pela revisão do manuscrito.

## FOOTNOTES

**a)** Período que medeia a transmissão do agente infeccioso após a sua ingestão por meio da refeição sanguínea.
**b)** Processo através do qual ixodídeos infectados e não infectados, se alimentam num determinado hospedeiro e em que a distância que os separa é inferior a um centímetro, o que os obriga a partilhar a mesma área alimentar.
**c)** Cada fase evolutiva parasita um hospedeiro diferente.
**d)** Um ciclo biológico ditrópico significa que as formas imaturas se alimentam-se num determinado tipo de hospedeiro, geralmente micromamíferos ou pequenos mamíferos silváticos, enquanto as formas adultas procuram hospedeiros de maior porte. No caso de ser monotrópico todas as fases evolutivas se alimentam-se no mesmo tipo de hospedeiro. Num ciclo politrópico todas as fases evolutivas têm uma grande capacidade de adaptação e podem-se alimentar-se em diversos hospedeiros.
**e)** Refere-se à dispersão da espécie relativamente ao local onde eclodiram. Assim formas adultas exofílicas significa que os adultos se podem afastar do local em que eclodiram ao contrário das formas endofilícas.
***f)*** Anaplasma phagocytophilum *é a actual designação para* Ehrlichia phagocytophila, E. equi *e agente da ehrlichiose granulocitica humana*[18].
**l)** Espécie também denominada por Boophilus annulatus

## BIBLIOGRAFIA

1 ARTHUR DR: Ticks and disease. Oxford: Pergamon Press 1962; 445.
2 SONENSHINE DE: Biology of ticks. Oxford: Oxford University, 1991; Vol.1 - 447.
3 SONENSHINE DE: Biology of ticks. Oxford: Oxford University, 1993; Vol.2 - 465.
4 REHACEK J: Ecological relationships between ticks and Rickettsiae. Eur J Epidemiol 1989;5(4):407-13.
5 REHACEK J: The role of ticks in dissemination of rickettsiae in nature. Acta Facult Rerum Natur Univers Comenianae – Zoologie 1991;35:127-30.
6. OMS: Artropod-borne and Rodent-borne Viral Diseases. Organização Mundial de Saúde, Geneva, 1985, serie nº 719.
7. GILOT B, LAFORGE ML, PICHOT J, RAOULT D: Relationships Between the *Rhipicephalus sanguineus* Complex Ecology and Mediterranean Spotted Fever Epidemiology in France. Eur J Epidemiol 1990;6(4):357-62.

8 GRESIKOVÁ M, SEKEYOVA M, STUPALOVA N, NECAS S: Sheep milk-borne epidemic of tick-borne encephalitis in Slovakia. Intervirology 1975;5:57-61.
9. CAEIRO V: General review of tick species present in Portugal. Parassitol 1999;41:11-5
10. DIAS JATS: As carraças (Acarina-Ixodoidea) da Península Ibérica. Algumas considerações sobre a sua Biogeografia e relacionamento com a ixodofauna Afropaleártica e Afrotropical. Lisboa: Estudos Ensaios e Documentos, Instituto de Investigação Científica Tropical 1994;163.
11. BACELLAR F, REGNERY RL, NÚNCIO MS, FILIPE AR: Genotypic evaluation of Rickettsial isolates recovered from various species of ticks in Portugal. Epidemiol Infect 1995;114:169--78.
12. BACELLAR F, SOUSA R, SANTOS A, SANTOS-SILVA M, PAROLA P: Boutonneuse fever in Portugal: 1995-2000. Data of a state laboratory. Eur J Epidemiol 2003;18:275-7.
13. SOUSA R, NOBREGA SD, BACELLAR F, TORGAL J: Sobre a realidade epidemiologica da febre escaro-nodular em Portugal. Act Med Port 2003;16:430-8.
14. POÇAS J, BACELLAR F, FILIPE AR: Clínica e diagnóstico laboratorial da febre escaro-nodular. Med Interna 2002;9:52-6.
15. FILIPE AR, CALISHER CH: Isolation of Thogoto virus from ticks in Portugal. Acta Virol 1984;28:152-5.
16. BACELLAR F: Rickettsias e Rickettsioses na Europa. In: Bettencourt A, Nobre G, eds. Arquivos do Instituto Câmara Pestana, 1998; 15-38.
17. NILSSON K, LINDQUIST O, PAHLSON C: Association of *Rickettsia helvetica* with chronic perimyocarditis in sudden death. Lancet 1999;354:1169-72.
18. DUMLER JS, BARBET AF, BEKKER CP, DASCH GA, PALMER GH, RAY SC et al.: Reorganization of genera in the families Rickettsiaceae and Anaplasmataceae in the order Rickettsiales: unification of some species of *Ehrlichia* with *Anaplasma*, *Cowdria* with *Ehrlichia* and *Ehrlichia* with *Neorickettsia*, descriptions of six new species combinations and designation of *Ehrlichia equi* and "HGE agent" as subjective synonyms of *Ehrlichia phagocytophila*. Int J Syst Evol Microbiol 2001;51(6):2145-65.
19. SANTOS AS, SANTOS-SILVA MM, ALMEIDA VC, BACELLAR F, DUMLER JS: Detection of *Anaplasma phagocytophilum* DNA in *Ixodes* ticks (Acari: Ixodidae) from Madeira Island and Setúbal District, Mainland Portugal. Emerg Infect Dis 2004;10(9):1643-8.
20. BLANCO JR, OTEO JA: Human granulocytic ehrlichiosis in Europe. Clin Microbiol Infect. 2002; 8:763-72.
21. SANTOS A, BACELLAR F, DAVID DE MORAIS J: Ehrlichiose granulocitica humana. Conceitos actuais. Rev Port Doenç Infecc 2004; Outubro-Dezembro: 12-7.
22. KUNZ CH: Tick-borne Encephalitis in Europe. Acta Leidensia 1992;60(2)1-2.
23. CAMACHO AT, PALLAS E, GESTAL JJ, GUITIÁN J, OLMEDA AS, KENNY M, TELFORD S: *Babesia microti*: uma nueva forma de babesiose humana na Europa? Enferm Infecc Microbiol Clin 2002;20(8):415-8.
24. BACELLAR F, NÚNCIO MS, ALVES MJ, FILIPE AR: *Rickettsia slovaca*: Un agente del grupo de las fiebres exantemáticas, en Portugal. Enferm Infecc. Microbiol Clin 1995;13:218-23
25. LAKOS A, RAOULT D: Tick-borne lymphadenopathy (TIBOLA) a *Rickettsia slovaca* infection? In: Raoult D, Brouqui P, eds. Rickettsiae and Rickettsial Diseases at the turn of the third millennium. Paris. Elsevier 1999; 258-61.
26. SANTOS-SILVA MM, FORMOSINHO P, MELO P, SANTOS A, FILIPE AR: Ixodídeos (Acari: Ixodidae) parasitas de aves em Portugal. Rev Port Ciênc Vet 2001;96(540):11-3.
27. BACELLAR F. Ticks and Rickettsiae in Portugal. In Raoult D, Brouqui P, eds. Rickettsiae and Rickettsial Diseases. Paris. Elsevier 1999;103-9.
28. Raoult D, Fournier PE, Abboud P, Caron F: First documented human *Rickettsia aeschlimannii* infection. Emerg Infect Dis 2002;8(7):748-9.
29. FILIPE AR, CASALS J: Isolation of Dhori from *Hyalomma margiatum* ticks in Portugal. Intervirology 1979;11:124-7.
30. FILIPE AR, ALVES MJ, KARABATSOS N, MATOS APA, NÚNCIO MS, BACELLAR F: PALMA virus, a new *Bunyaviridae* isolated from ticks in Portugal. Intervirology 1994;37:348-51.
31. LABUDA M, ALVES MJ, ELECKOVÁ E, FILIPE AR: Transmission of tick-borne Bunyaviruses by cofeeding ixodid ticks. Acta Virol 1997;41:325-8.
32. BAPTISTA S, QUARESMA A, AIRES T, KURTENBACH K, SANTOS-REIS M, NICHOLSON M, COLLARES-PEREIRA M: Lyme borreliosis spirochetes in questing ticks from mainland Portugal. Int J Med Microbiol 2004; 293(37)109-16.
33. NÚNCIO MS, PÉTER O, ALVES MJ, BACELLAR F, FILIPE AR: Isolamento e caracterização de borrélias de Ixodes ricinus L. Em Portugal. Rev Port Doenç Infecc 1993; 16(3):175-9.


48

# APPENDIX 2. EHRLICHIOSE GRANULOCÍTICA HUMANA: CONCEITOS ACTUAIS

SANTOS AS, BACELLAR F, DAVID DE MORAIS J

REVISION ARTICLE

REVISTA PORTUGUESA DE DOENÇAS INFECCIOSAS 2004; OUTUBRO/DEZEMBRO:12-17

ARTIGO ORIGINAL
ORIGINAL ARTICLE

# Ehrlichiose Granulocítica Humana
## Conceitos actuais

A. Santos[1], F. Bacellar[1], J. David de Morais[2]

1 Centro de Estudos de Vectores e Doenças Infecciosas, Instituto Nacional de Saúde Dr. Ricardo Jorge, Águas de Moura.
2 Departamento de Ecologia, Universidade de Évora, Évora.

### EHRLICHIOSE HUMANA

A ehrlichiose é uma doença infecciosa tradicionalmente associada aos animais, que afecta diversos mamíferos domésticos e silváticos. É conhecida há mais meio século, desde a descrição da ehrlichiose canina em 1935[1,2] e da ehrlichiose bovina e ovina em 1936[3,4]. Nas últimas décadas, esta doença tem merecido um interesse crescente em medicina humana, o que veio conduzir a intensas investigações nesta área até então considerada exclusivamente do âmbito veterinário.

Em termos etiológicos, a ehrlichiose é causada por bactérias Gram negativas intracelulares obrigatórias, que se desenvolvem no interior dos vacúolos fagossómicos de células eucariotas[5]. O ciclo de transmissão destes microrganismos inclui a infecção tanto de artrópodes vectores (essencialmente carraças) como de hospedeiros vertebrados. Nestes últimos, são infectadas predominantemente as células de origem hematopoiética, com excepção dos eritrócitos. Em termo taxonómicos, estes microrganismos foram reunidos num único grupo: o género *Ehrlichia* (pertencente à tribo Ehrlichia-eae, família Rickettsiaceae, ordem Rickettsiales). Estudos filogenéticos, com base em técnicas de biologia molecular, têm vindo a demonstrar a sua heterogeneidade e a proximidade de alguns dos seus membros com outros microrganismos. Assim, foi proposta a reclassificação de algumas ehrlichias no género *Neorickettsia* e no género *Anaplasma*, mantendo-se no género *Ehrlichia* apenas algumas das espécies originalmente descritas[6] (vide Quadro I). Compreender-se-á, pois, que quando hoje em dia falamos de "ehrlichioses" estamos, na verdade, do ponto de vista etiológico, a utilizar um termo que inclui afinal ehrlichioses propriamente ditas mas também anaplasmoses humanas, designativo este que, em rigor, deveríamos utilizar, e que por certo terá tendência a impor-se no futuro.

Embora, o primeiro caso de ehrlichiose humana (febre de Sennetsu) tenha sido documentado no Japão, em 1954,[7] a doença foi considerada de reduzida importância médica. Na verdade, esta ehrlichiose causada por *Neorickettsia* (*Ehrlichia*) *sennetsu* é pouco frequente e de gravidade moderada, não havendo registos de mortalidade associados, ocorrendo apenas em áreas limitadas do Extremo Oriente, raramente fora do Japão.

Só nas últimas décadas do século XX, com a descoberta de outras ehrlichias associadas a patologias humanas de gravidade variável no Ocidente, é que foi radicalmente alterada a forma como a comunidade científica encarava a problemática da ehrlichiose humana.

Em 1986, foi diagnosticada pela primeira vez a ehrlichiose monocítica (ou monocitotrópica) humana (EMH), nos E.U.A..[8] Esta doença foi associada a uma nova espécie, designada por *Ehrlichia chaffeensis*.[9,10] Mais tarde, em 1994, foram descritos os primeiros casos de ehrlichiose granulocítica (ou granulocitotrópica) humana (EGH).[11] Como a identidade exacta do agente envolvido nesta doença era desconhecida, foi adoptada a designação de "agente da EGH". Em 1996, foram descritos na Venezuela casos de infecção humana por um agente semelhante a *Ehrlichia canis* (agente etiológico da ehrlichiose monocítica canina).[12] Mais recentemente, em 1999, também *Ehrlichia ewingii* (conhecido agente da ehrlichiose granulocítica canina) foi implicada em casos de ehrlichiose humana nos E.U.A..[13]

Actualmente, a EMH e a EGH são consideradas as ehrlichioses humanas mais importantes em Saúde Pública, não só pela sua alargada distribuição geográfica, mas também pela casuística que lhes está associada. Estas ehrlichioses ocorrem essencialmente nos Estados Unidos, havendo também registos da sua presença noutros pontos do globo. Na Europa, existem referências de infecção humana por um agente imunologicamente semelhante a *E. chaffeensis* em Portugal,[14] Espanha[15] e Bélgica.[16] Porém, estudos recentes apontam para que seja a EGH a ter um papel de relevo nos países europeus.

Com a presente revisão da EGH pretendemos alertar os clínicos para os principais aspectos epidemiológicos e clínicos, bem como para o diagnóstico e tratamento desta ehrlichiose, à semelhança do que já havia sido feito, por um de nós, para a EMH.[17]

### EHRLICHIOSE GRANULOCÍTICA HUMANA

#### ETIOLOGIA

Como já foi referido, a ehrlichiose granulocítica humana é causada por um agente que permaneceu, até há bem pouco tempo, sem nomenclatura oficial, tendo sido designado por agente da EGH. Nos anos seguintes ao seu isolamento, foram reunidos dados genéticos,[11,18] antigénicos[19,20] e biológicos que sugeriam a existência de uma estreita relação com *Ehrlichia phagocytophila* (agente da febre da carraça dos ruminantes) e *Ehrlichia equi* (agente da ehrlichiose granulocítica equina), defenden-

do-se que representariam diferentes estirpes de uma única espécie. Actualmente essa hipótese está confirmada, e além disso há evidências da proximidade genética entre estes microrganismos e os anaplasmas, o que levou a que fosse proposta a sua reclassificação no Género *Anaplasma*, como uma única espécie, com a designação de *Anaplasma phagocytophila*[6,21](vide Fig. I).

## EPIDEMIOLOGIA

Desde que foi descrita pela primeira vez no Minnesota e Wisconsin, em 1994, a EGH têm vindo a ser sucessivamente identificada em diversos estados americanos, apresentando actualmente uma vasta distribuição geográfica nos Estados Unidos. Ao contrário da EMH, que está confinada à região Sul e Sudoeste, esta ehrlichiose ocorre predominantemente na região Nordeste e Noroeste do País, apresentando nas áreas endémicas uma frequência semelhante à verificada para a borreliose de Lyme.[22] O principal ixodídeo vector é *Ixodes scapularis*, também implicado na transmissão de *Borrelia burgdorferi* (agente etiológico da borreliose de Lyme) e de *Babesia microti* (associada a casos de babesiose humana). O ciclo epidemiológico do agente envolve pequenos roedores e ruminantes silváticos, principalmente veados, que servem de hospedeiros vertebrados aos artrópodes vectores.[23,24,25,26,27] Esta doença afecta um largo espectro de mamíferos, incluindo equídeos, ruminantes, canídeos e o Homem.[20,28,29,30,31] A infecção humana está associada ao contacto com o ixodídeo vector. No entanto, a descrição de um caso de infecção perinatal[32] e a associação da doença à manipulação de carne fresca de cervídeos[33] realça a existência de vias alternativas de infecção.

O crescente interesse por este tema e o desenvolvimento de linhas de investigação a nível mundial revelaram evidências significativas de que EGH não está confinada apenas aos Estados Unidos, ocorrendo também noutros países, principalmente da Europa. O contacto com *A. pahagocytophila* (estirpe agente da EGH) foi detectado em estudos serológicos realizados no Reino Unido,[34] Suíça,[35] Noruega,[36] Suécia[37] e Espanha.[38] Estudos moleculares também identificaram a presença de ADN específico do agente em carraças *Ixodes ricinus* capturadas na Itália,[39] Suécia,[40] França,[41] Eslovénia,[42] Alemanha,[43] Holanda,[44] Suíça,[45] Portugal[46] e Espanha[38]. Há também uma referência à detecção de ADN específico em ácaros *Neotrombicula autumnalis* em Espanha.[47] Estes dados sugerem que *A. phagocytophila* apresenta uma larga distribuição geográfica na Europa, partilhando o mesmo biótopo que o agente da borreliose de Lyme e sendo transmitido pelo mesmo ixodídeo vector (*I. ricinus*). Embora a sua associação a patologias humanas seja, até à data, reduzida,[48,49] julga-se provável que esta realidade possa estar subavaliada devido possivelmente à falta de sensibilização dos clínicos e à escassez de laboratórios com o diagnóstico específico.

## CLÍNICA

O tempo médio de incubação da ehrlichiose humana cifra-se em cerca de oito dias.[50]

Comparativamente com a ehrlichiose monocítica, a ehrlichiose granulocítica costuma cursar com quadros clínicos mais moderados (vide Quadro II). A sintomatologia mais frequente da EGH inclui febre, arrepios, mal-estar geral, cefaleias e mialgias, podendo estas ser muito intensas. Cerca de metade dos doentes podem também apresentar anorexia, náuseas, vómitos, dores abdominais, diarreia, tosse e artralgias.[51] Em menos de 10% dos casos pode coexistir *rash* cutâneo.[52,53] Embora raramente, o quadro clínico pode evoluir para rabdomiólise, miocardite, falência respiratória aguda, *shock* séptico, polineuropatia desmielinizante, meningite asséptica e meningoencefalite.[52,53,54,55,56,57,58] Os casos mais graves e que requerem períodos mais prolongados de internamento hospitalar respeitam muitas vezes a indivíduos idosos, diabéticos, pacientes com doenças vasculares do colagénio, imunodeprimidos e casos de diagnóstico tardio de ehrlichiose.[51] Estão também referidas complicações importantes do quadro clínico em indivíduos a quem foi administrada terapêutica antibiótica prévia com co-trimoxazol, como aliás acontece nas ricketsioses em geral.[59]

A mortalidade na EGH cifra-se entre 0,5 e 1%, enquanto na EMH costuma ser mais elevada: 2,7%.[51]

## DIAGNÓSTICO

A hipótese de ehrlichiose deverá ser posta face a quadros febris de origem incerta com:

- história de picada de ixodídeo e/ou exposição a ixodídeos em área endémica nas duas semanas prévias;
- existência de trombocitopenia e leucopenia;
- aumento dos valores das transaminases séricas.

Nestes casos os testes laboratoriais são imprescindíveis, visto ser virtualmente

impossível o diagnóstico definitivo da EGH apenas com base em aspectos epidemiológicos e clínicos.[60] Os testes laboratoriais disponíveis são de dois tipos: directos, que visam a detecção do agente (exame microscópico, análise molecular e isolamento) ou indirectos, que detectam anticorpos produzidos na sequência da infecção (análises sero-imunológicas).[61]

Como *A. phagocytophila* infecta células de origem mielocítica, apresentando um marcado tropismo por neutrófilos, sangue total com anti-coagulante (preferencialmente sem heparina) e aspirado medular (se clinicamente apropriado) são as amostras mais adequadas para a realização dos testes directos. Este material biológico deve ser obtido durante a fase aguda da doença, antes da instituição da terapêutica antibiótica. As amostras devem ser refrigeradas a 4ºC, mantidas no recipiente de colheita para garantir condições de esterilidade (situação indispensável caso se pretenda fazer o isolamento do agente) e enviadas no frio para o laboratório (idealmente nas 24h subsequentes à colheita). As amostras de soro ou plasma dos doentes destinam-se apenas aos testes imunológicos. É importante salientar que 60% dos doentes com suspeita de ehrlichiose apresentam resultados serológicos negativos na fase aguda da doença, verificando-se que mais de 80% destes casos desenvolvem posteriormente níveis de anticorpos detectáveis.[60] O estudo da cinética dos anticorpos da EGH tem mostrado que o aparecimento de imunoglobulinas específicas ocorre durante a segunda semana de doença, verificando-se uma elevação dos títulos até às quatro a seis semanas, permanecendo elevados durante vários meses.[61,62] É pois recomendável para os testes indirectos o envio de duas amostras de soro/plasma do doente: a primeira, o mais precocemente possível, seguida de uma segunda amostra colhida 4 a 8 semanas depois.[60]

O exame microscópico é realizado por observação dos esfregaços de sangue periférico corados por Giemsa ou *Diff Quick* para pesquisa de agregados bacterianos intra-leucocitários (mórulas). No entanto, devido à leucopenia que frequentemente é observada durante a fase aguda da EGH e ao baixo nível de infecção celular é recomendável a realização de uma concentração leucocitária e a observação de 800-1000 granulócitos por esfregaço.[61] Todavia, importará referir que, nos EUA, a detecção de mórulas na primeira semana, só ocorreu em cerca de 62% dos casos de EGH63 e que na Europa ela tem sido bastante mais rara.[63] Acrescenta-se ainda a possibilidade de ocorrerem falso positivos. A presença de granulações intra-leucocitárias (como granulações tóxicas ou corpos de Döhle) e a sobreposição de plaquetas ou partículas contaminantes poderão ser confundidas com mórulas de ehrlichia.[60] Assim, o exame microscópico não deverá por si só ser suficiente para confirmar o diagnóstico de EGH.

A análise molecular é uma alternativa. A reacção de polimerase em cadeia (PCR) é a técnica molecular de eleição para o diagnóstico da EGH. A sensibilidade desta técnica varia entre 67-87% e a especificidade é próxima dos 100%.[63] Existem diversos pares de oligonucleotidos que podem ser usados na detecção das estirpes de *A. phagocytophila* por PCR. Na sua grande maioria amplificam segmentos do gene que codifica a sub-unidade 16S do ARN ribossomal ou a região do operão *gro*ESL.11,18

O isolamento do agente é ainda outra técnica directa para confirmar o diagnóstico de EGH. A cultura de ehrlichias é correntemente realizada em células HL-60,[64] mas esta técnica só está disponível em laboratórios especializados. Demais, o crescimento das ehrlichias poderá levar vários dias ou até mais de duas semanas, tornando moroso o resultado da confirmação laboratorial.

Em termos práticos, é pois nas análises sero-imunológicas que muitas vezes assenta o diagnóstico definitivo de ehrli-

**Quadro I - Classificação actual e principais aspectos epidemiológicos dos agentes tradicionalmente associados a ehrlichioses**

| Agente etiológico | Doença | Vectores/Hospedeiros | Tropismo celular |
|---|---|---|---|
| **Género *Ehrlichia*** | | | |
| *E. canis* | Ehrlichiose monocítica canina | Ixodídeos/Canídeos | Leucócitos mononucleares |
| *E. chaffeensis* | Ehrlichiose monocítica humana | Ixodídeos/Cervídeos, canídeos e homem | Leucócitos mononucleares |
| *E. ewingii* | Ehrlichiose granulocítica canina | Ixodídeos/Canídeos e homem | Leucócitos polimorfonucleares |
| *E. muris* | Provoca esplenomegália | Ixodídeos/Roedores | Leucócitos mononucleares |
| **Género *Anaplasma*** | | | |
| *A. phagocytophila* (*Ehrlichia phagocytophila*) | Febre da carraça dos ruminantes | Ixodídeos/Ruminantes | Leucócitos polimorfonucleares |
| *A. phagocytophila* (*Ehrlichia equi*) | Ehrlichiose granulocítica equina | Ixodídeos/Equídeos | Leucócitos polimorfonucleares |
| *A. phagocytophila* (agente da EGH) | Ehrlichiose granulocítica humana | Ixodídeos/Ruminantes, equídeos, canídeos e homem | Leucócitos polimorfonucleares |
| *A. bovis* (*Ehrlichia bovis*) | Ehrlichiose bovina | Ixodídeos/Ruminantes | Leucócitos mononucleares |
| *A. platys* (Ehrlichia platys) | Trombocitopenia cíclica infecciosa dos canídeos | Ixodídeos/Canídeos | Plaquetas |
| **Género *Neorickettsia*** | | | |
| *N. sennetsu* (*Ehrlichia sennetsu*) | Febre de Sennetsu | Tremátodos/Homem | Leucócitos mononucleares |
| *N. risticii* (*Ehrlichia risticii*) | Síndroma diarreíco agudo dos equídeos | Tremátodos/Equídeos | Leucócitos mononucleares |

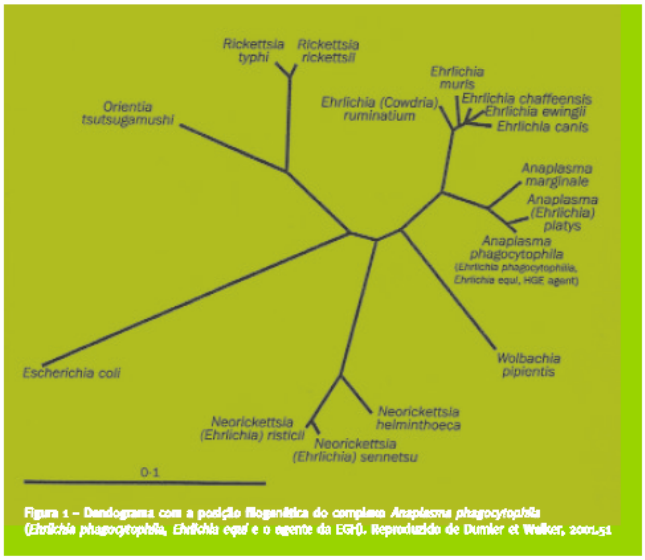



**Figura 1 – Dendograma com a posição filogenética do complexo *Anaplasma phagocytophila* (*Ehrlichia phagocytophila*, *Ehrlichia equi* e o agente da EGH). Reproduzido de Dumler et Walker, 2001.51**

chiose. A pesquisa de imunoglobulinas das classes M e G por imunofluorescência indirecta (IFA) é a técnica aconselhada.[60] Devem ser usados como antigénio isolados de *A. phagocytophila* (estirpes *E. phagocytophila*, *E. equi* ou agente da EGH) e o limiar de positividade (diluição a partir da qual um soro deve ser considerado positivo) deve ser determinado por cada laboratório.[60] A sensibilidade da IFA nos casos de EGH confirmados por exame cultural é superior a 95%.[61] Outros testes serológicos como ELISA e Imunoblot, com microrganismos purificados ou com proteínas recombinantes, estão a ser desenvolvidos e tudo indica que a curto prazo possam vir a ser aplicados correntemente no diagnóstico da EGH.[67,68,69,70]

A confirmação laboratorial de um caso de EGH, com diagnóstico clínico compatível, é definida por: (i) seroconversão; (ii) alteração do título de anticorpos de pelo menos quatro vezes, relativamente ao título do soro inicial; (iii) detecção de mórulas no exame microscópico, associado a PCR positivo; (iv) isolamento do agente em cultura celular. Considera-se ainda como caso provável a detecção de mórulas intra-leucocitárias ou a presença de uma única amostra de soro com título superior ao limiar de positividade.[60,62,71]

O Centro de Estudos de Vectores e Doenças Infecciosas (CEVDI) dispõe actualmente do teste de IFA para a detecção de infecções pelo agente EGH. Nesta análise é utilizado antigénio comercial (# IF 1402, Focus Technologies), bem como antigénio fornecido Prof. Stephen Dumler, da Divisão de Microbiologia Médica do Departamento de Patologia, Johns Hopkins Medical Institutions, EUA.

## TRATAMENTO

Para além da terapêutica sintomática eventualmente necessária, o tratamento de escolha da ehrlichiose assenta na prescrição da doxiciclina. A dose habitualmente recomendada é de 100 mg, 2 vezes/dia, durante uma semana, ou mais três a cinco dias após a defervescência da febre. Todavia, em áreas endémicas de borreliose de Lyme, o tratamento deverá ser mantido, em princípio, durante duas a três semanas, dada a possibilidade de existência de infecção simultânea pelas duas zoonoses.[43,72] Uma vez que o diagnóstico laboratorial poderá demorar algum tempo, frente a um caso de suspeita de ehrlichiose a terapêutica antibiótica apropriada deverá ser logo instituída.[73]

*In vitro*, as ehrlichias mostraram-se também sensíveis à rifamicina e à rifampicina, sendo este último antibiótico habitualmente preferido em grávidas infectadas.[74]

As ehrlichias não evidenciaram sensibilidade às cefalosporinas, aminoglicosídeos, macrólidos e cloranfenicol,[34,52] pelo que são questionáveis alguns pretensos bons resultados obtidos com tais antibióticos. Recorde-se que nos casos mais frustres de ehrlichiose a sintomatologia pode regredir espontaneamente sem qualquer terapêutica, além de poderem existir casos assintomáticos, como o atestam os inquéritos sero-epidemiológicos.

Como já antes dito, face a um diagnóstico presuntivo de ehrlichiose a terapêutica com co-trimoxazol deverá ser completamente banida.

## COMENTÁRIO FINAL

No caso concreto de Portugal, a descoberta do agente da EGH em território nacional e a sua associação ao ixodídeo vector implicado na transmissão da *B. burgdorferi*46 poderá ter amplas implicações em Saúde Pública.

A borreliose de Lyme só agora começou a merecer mais atenção por parte das entidades oficiais, sendo desde 1999 uma doença de declaração obrigatória.[75] Contudo, do universo de episódios febris supostamente relacionados com a borreliose de Lyme, apenas numa parte dos casos este diagnóstico é confirmado laboratorialmente.[76,76] Assim, e à luz desta nova descoberta, é possível que algumas dessas situações "não conclusivas" sejam de facto resultantes da infecção humana por este novo agente. Deverá também ser considerada a possibilidade de infecções conjuntas do agente EGH e de *B. burgdorferi*, à semelhança do que se verifica noutros países onde estes dois agentes coexistem. [77,78,79,80]

## CORRESPONDÊNCIA

Ana Sofia Santos. Centro de Estudos de Vectores e Doenças Infecciosas, Instituto Nacional de Saúde Dr. Ricardo Jorge. 2965 Águas de Moura, Portugal.
Telef. 265 912 222; Fax. 265 912 155;
e-mail: ana.santos@insa.min-saude.pt

## SUMÁRIO

A ehrlichiose humana representa um dos melhores exemplos de doenças infecciosas emergentes, com a ehrlichiose granulocítica humana (EGH) a merecer o papel de maior destaque na Europa. Esta doença é causada por um conhe-

cido agente de interesse veterinário *Anaplasma (Ehrlichia) phagocytophila*. Estudos recentes demonstraram que este agente ocorre em vários países europeus, designadamente Portugal, partilhando a mesma área geográfica do agente da borreliose de Lyme, e sendo transmitido pelo mesmo ixodídeo vector (*Ixodes ricinus*). Embora o número de casos de infecção humana por este agente seja até à data reduzido, os autores discutem o facto desta zoonose poder estar subavaliada devido possivelmente à falta de sensibilização dos clínicos e à escassez de laboratórios equipados para o diagnóstico específico. Este artigo aborda os principais aspectos epidemiológicos, clínicos, de diagnóstico e tratamento da EGH. Os autores pretendem também divulgar que o CEVDI tem à disposição técnicas serológicas para o diagnóstico laboratorial de EGH.

**Quadro II**
**Principais aspectos epidemiológicos, clínico e laboratoriais distintivos das duas principais ehrlichioses humanas**

| | EGH | EMH |
|---|---|---|
| **Agente etiológico** | *Anaplasma phagocytophila* | *Ehrlichia chaffeensis* |
| **Vector** | *Ixodes* spp. | *Amblyomma americanum*<br>*Dermacentor variabilis* |
| **Principais hospedeiros** | Homem, cervídeos, roedores, cavalos, cães | Homem, cervídeos, cães |
| **Principal sintomatologia** | Febre, mal-estar geral, cefaleias, mialgias | Idem, rash cutâneo, falência respiratória aguda, falência renal aguda, meningoencefalite |
| **Mortalidade** | 0,5 a 1% | 2,7% |
| **Tropismo celular** | Neutrófilos | Monócitos/macrófagos |
| **Presença de mórulas** | 10 a 73% | 0 a 1% |
| **Cultura de células** | HL60 | DH82 |
| **Imunoblot** | 25, **40**, 42, **44**, 46, **65**, 110 kD | 22, 25, **27**, **29**, **44**, 55, 66, 120 kD |

Quadro adaptado de Bakken et Dumler, 2000 e Dumler et Walker, 2001.51,63

**ABSTRACT**

Human ehrlichiosis represent one of the best examples of newly emergent infectious diseases with human granulocytic ehrlichiosis (HGE) standing out as the most relevant in Europe. This disease is caused by a well-known veterinary pathogen *Anaplasma (Ehrlichia) phagocytophila*. Recent studies suggest that this agent may occur broadly across several European countries, including Portugal, within the same geographic range as the Lyme borreliosis agent and sharing the same vector tick (*Ixodes ricinus*). Although the number of human infection is few, the authors discuss the possibility that this zoonotic disease could be underestimated due to a lack of physician awareness and unavailability of current laboratory diagnosis. This article focuses on the epidemiology, clinical manifestations, diagnosis and treatment of HGE. The authors also point out that CEVDI has already available serologic tests for the diagnosis of HGE.

**BIBLIOGRAFIA**

1 - Donatien A, Lestoquard F. Existence en Algérie d'une *Rickettsia* du chien. *Bull Soc Path Exot* 1935; 27: 418-9.
2 - Donatien A, Lestoquard F. État Actuel des Connaissances sur les Rickettsioses Animales. *Archives de l'Institut Pasteur d'Algérie* 1937; 15(2): 162-77.
3 - Donatien A, Lestoquard F. *Rickettsia bovis*, novelle espèce pathogène pour le bœuf. *Bull Soc Path Exot* 1936 ; 29 : 1057-61.
4 - Donatien A, Lestoquard F. Sur une nouvelle rickettsia du mouton. *Bull Soc Path Exot* 1936; 29 : 105-8.
5 - Ristic M, Huxsoll DL. Tribe II. *Ehrlichiae* Philip 1957. In: Krieg NR, Holt JG (eds.). *Bergey's Manual of Systematic Bacteriology*, vol. I. Baltimore: Williams & Wilkins; 1984; p, 704-11.
6 - Dumler JS, Barbet AF, Bekker CP, Dasch GA, Palmer GH, Ray SC, et al. Reorganization of genera in the families Rickettsiaceae and Anaplasmataceae in the order Rickettsiales: unification of some species of *Ehrlichia* with *Anaplasma*, *Cowdria* with *Ehrlichia* and *Ehrlichia* with *Neorickettsia*, descriptions of six new species combinations and designation of *Ehrlichia equi* and "HGE agent" as subjective synonyms of *Ehrlichia phagocytophila*. *Int J Syst Evol Microbiol* 2001; 51(6): 2145-65.
7 - Misao T, Kobayashi Y. Studies on infectious mononucleosis (glandular fever). I. Isolation of etiologic agent from blood, bone marrow and lymph node of a patient with infectious mononucleosis by using mice. *Kyushu J Med Sci* 1955; 6:145-52.
8 - Maeda K, Markowitz N, Hawley RC, Ristic M, Cox D, McDade JE. Human infection with *Ehrlichia canis*, a leukocytic rickettsia. *N Eng J Med* 1987; 316: 853-6.
9 - Dawson JE, Anderson BE, Fishbein DB, Sanchez JL, Goldsmith CS, Wilson KH, et al. Isolation and characterization of an *Ehrlichia sp.* from a patient diagnosed with human ehrlichiosis. *J Clin Microbiol* 1991; 29(12): 2741-5.
10 - Anderson BE, Dawson JE, Jones DC, Wilson KH. *Ehrlichia chaffeensis*, a new species associated with human ehrlichiosis. *J Clin Microbiol* 1991; 29(12): 2838-42.
11 - Chen S-M, Dumler JS, Bakken JS, Walker DH. Identification of a granulocytotropic ehrlichia species as the etiologic agent of human diseases. *J Clin Microbiol* 1994; 32(3): 589-95.
12 - Perez M, Rikihisa Y, Wen B. Ehrlichia canis-like agent isolated from a man in Venezuela: antigenic and genetic characterization. *J Clin* Microbiol 1996; 34(9): 2133-9.
13 - Buller RS, Arens M, Hmiel SP, Paddock CD, Sumner JW, Rikihisa Y, et al. *Ehrlichia ewingii*, a newly recognized agent of human ehrlichiosis. *N Engl J Med* 1999; 341(3): 148-55.
14 - David de Morais JA, Dawson JE, Greene C, Filipe AR, Galhardas LC, Bacellar F. First european case of ehrlichiosis. *Lancet* 1991; 338: 633-4.
15 - Saz JV, Dawson JE, Bacellar F, Merino FJ, Filipe A. A propósito de la ehrlichiosis humana en España. *Enfermedades Infecciosas e Microbiologia Clinica* 1994; 7(7): 357-8.
16 - Pierard D, Levtchenko L, Dawson JE, Lauwers S. Ehrlichiosis in Belgium. *Lancet* 1995; 346: 1233-4.
17 - David de Morais JA. Epidemiologia e clínica da ehrlichiose humana. A propósito do primeiro caso diagnosticado na Europa. *Rev Port Doenças Infec* 1992; 15(1): 7-18.
18 - Sumner JW, Nicholson WL, Massung RF. PCR amplification and comparison of nucleotide sequences from the groESL heat shock operon of *Ehrlichia* species. *J Clin Microbiol* 1997; 35(8): 2087-92.
19 - Dumler JS, Asanovich KM, Bakken JS, Richter P, Kimsey R, Madigan JE. Serologic cross-reactions among *Ehrlichia equi*, *Ehrlichia phagocytophila* and human Granulocytic Ehrlichia. *J Clin Microbiol* 1995; 33(5): 1098-103.
20 - Barlough JE, Madigan JE, DeRock E, Dumler JS, Bakken JS. Protection against *Ehrlichia equi* is conferred by prior infection with the human granulocytotropic ehrlichia (HGE agent). *J Clin Microbiol* 1995; 33(12): 3333-34.
21 - Yu X-J, Zhang X-F, McBride JW, Zhang Y, Walker D. Phylogenetic relationships of *Anaplasma marginale* and "*Ehrlichia platys*" to other *Ehrlichia* species determined by GroEL amino acid sequences. *Int J Syst Evol Microbiol* 2001; 51: 1143-6.
22 - Dumler JS. The ehrlichioses: an overview. *Infect Dis Review* 1999; 1(2):110-2.
23 - Telford SR, Dawson JE, Katavolos P, Warner CK, Kolbert CP, Persing DH. Perpetuation of the agent of human granulocytic ehrlichiosis in a deer tick-rodent cycle. *Proc Natl Acad Sci USA* 1996; 93: 6209-14.
24 - Belongia EA, Reed KD, Mitchell PD, Kolbert CP, Persing DH, Gill JS, et al. Prevalence of granulocytic *Ehrlichia* infection among white-tailed deer in Wisconsin. *J Clin Microbiol* 1997; 35(6): 1465-68.
25 - Walls JJ, Greig B, Neitzel DF, Dumler JS. Natural infection of small mammal species in Minnesota with the agent of human granulocytic ehrlichiosis. *J Clin Microbiol* 1997; 35(4): 853-5.

26 - Nicholson WL, Muir S, Sumner JW, Childs JE. Serologic evidence of infection with *Ehrlichia spp.* in wild rodents (Muridae: Sigmodontinae) in the United States. *J Clin Microbiol* 1998; 36(3): 695-700.
27 - Nicholson WL, Castro MB, Kramer VL, Sumner JW, Childs JE. Dusky-footed wood rats (*Neotoma fuscipes*) as reservoirs of granulocytic ehrlichiae (Rickettsiales: Ehrlichieae) in Northern California. *J Clin Microbiol* 1999; 37(10): 3323-7.
28 - Greig B, Asanovich KM, Armstrong PJ, Dumler, JS. Geographic, clinical, serologic and molecular evidence of Granulocytic ehrlichiosis, a likely zoonotic disease, in Minnesota and Wisconsin dogs. *J Clin Microbiol* 1996; 34(1): 44-8.
29 - Madigan JE, Barlough JE, Dumler JS, Schankman NS, DeRock E. Equine granulocytic ehrlichiosis in Connecticut caused by an agent resembling the human granulocytotropic ehrlichia. *J Clin Microbiol* 1996; 32(2): 434-5
30 - Pusterla N, Huder J, Wolfensberger C, Litschi B, Parvis A, Lutz H. Granulocytic ehrlichiosis in two dogs in Switzerland. *J Clin Microbiol.* 1997; 35(9): 2307-9.
31 - Pusterla N, Pusterla JB, Deplazes P, Wolfensberger C, Muller W, Horauf A, et al. Seroprevalence of *Ehrlichia canis* and Canine granulocytic ehrlichia infection in Dogs in Switzerland. *J Clin Microbiol* 1998; 36(12): 3460-2.
32 - Horowitz HW, Kilchevsky E, Haber S, Aguero-Rosenfeld M, Kranwinkel R, James EK, et al. Perinatal transmission of the agent of human granulocytic ehrlichiosis. *N Engl J Med* 1998; 339: 375-8.
33 - Bakken JS, Krueth J, Lund T, Malkovitch D, Asanovich K, Dumler JS. Exposure to deer blood may be a cause of human granulocytic ehrlichiosis. *Clin Infect Dis* 1996; 23: 198.
34 - Sumption KJ, Wright DJ, Cutler SJ, Dale BA. Human ehrlichiosis in the UK. *Lancet* 1995; 346(2): 1487-88.
35 - Brouqui PH, Dumler JS, Lienhard R, Brossard M, Raoult D. Human granulocytic ehrlichiosis in Europe. *Lancet* 1995; 346(16):782-3.
36 - Bakken JS, Krueth J, Tilden RL, Dumler JS, Kristiansen BE. Serological evidence of human granulocytic ehrlichiosis in Norway. *Eur J Microbiol Infect Dis* 1996; 15: 829-32.
37 - Dumler JS, Dotevall L, Gustafson R. A Population–based seroepidemiologic study of human granulocytic ehrlichiosis and Lyme borreliosis on the west coast of Sweden. *J Infect Diseases* 1997; 175: 720-2.
38 - Oteo JA, Gil H, Barral M, Pérez A, Jimenez S, Blanco JR, et al. Presence of granulocitic ehrlichia in ticks and serological evidence of human infection in La Rioja, Spain. *Epidemiol Infect* 2001; 127: 353-8.
39 - Cinco M, Padovan D, Murgia R, Maroli M, Frusteri L, Heldtander M, et al. Coexistence of *Ehrlichia phagocytophila* and *Borrelia burgdorferi sensu lato* in *Ixodes ricinus* ticks Italy as determined by 16S rRNA gene sequencing. *J Clin Microbiol* 1997; 35:3365-66.
40 - Von Stedingk LV, Gurtelschmid M, Hanson HS, Gustafson R, Dotevall L, Engvall EO, et al. The human granulocytic ehrlichiosis (HGE) agent in Swedish ticks. *Clin Microbiol Infect* 1997; 3:573-4.
41 - Parola P, Beati L, Cambon M, Brouqui P, Raoult D. Ehrlichial DNA amplified from *Ixodes ricinus* (Acari: Ixodidae) in France. *J Med Entomol* 1998; 35: 180-3.
42 - Petrovec M, Sumner JW, Nicholson WL, Childs JE, Strle F, Barlic J, et al. Identity of ehrlichial DNA sequences derived from *Ixodes ricinus* ticks with those obtained from patients with human granulocytic ehrlichiosis in Slovenia. *J Clin Microbiol* 1999; 37(1): 209-10.
43 - Baumgarten BU, Rollinghoff M, Bogdan C. Prevalence of *Borrelia burgdorferi* and granulocytic and monocytic ehrlichiae in *Ixodes ricinus* ticks from southern Germany. *J Clin Microbiol* 1999; 37(11): 3448-51.
44 - Schouls LM, Van De Pol I, Rijpkema SGT, Schot CS. Detection and identification of *Ehrlichia, Borrelia burgdorferi sensu lato*, and *Bartonella* species in dutch *Ixodes ricinus* ticks. *J Clin Microbiol* 1999; 37(7): 2215-22.
45 - Pusterla N, Leutenegger CM, Huder JB, Weber R, Braun U, Lutz H. Evidence of human granulocytic ehrlichiosis agent in *Ixodes ricinus* ticks in Switzerland. *J Clin Microbiol* 1999; 37(5):1332-4.
46 - Núncio MS, Schouls LM, Van De Pol I, Bacellar F, Filipe A. The role of *Ixodes ricinus* as a vector of zoonotic agents in Madeira Island. Oxford: Seventh Bienal Meeting of European Tick Study Group; 28 de Abril 2000.
47 - Fernández-Soto P, Manzano R, García-Varas S, Madrazo C, Pérez-Sánchez R, Encinas A. Las larvas de *Neotrombicula autumnalis* son probables vectores de *Ehrlicia phagocytophila. Acta Parasitológica Portuguesa* 2001; 8: 325.
48 - Petrovec M, Furlan SL, Zupanc TA, Strle F, Brouqui P, Roux V, et al. Human disease in Europe caused by a granulocytic *Ehrlichia* species. *J Clin Microbiol* 1997; 35(6): 1556-9.
49 - Lotric-Furlan S, Petrovec M, Zupanac TA, Nicholson WL, Sumner JW, Childs JE, et al. Human granulocytic ehrlichiosis in Europe: clinical and laboratory findings for four patients from Slovenia. *Clin Infect Dis* 1998; 27: 424-8.
50 - Walker DH, Dumler JS. Emergence of the ehrlichiose as human health problems. *Emerg Infect Dis* 1996; 2: 18-28.
51 - Dumler JS, Walker DH. Tick-borne ehrlichioses. *Lancet Infectious Diseases*, April 2001: 218.
52 - Aguero-Rosenfeld ME, Horowitz HW, Wormser GP, Mckenna DF, Nowakowski J, Muñoz J. Human granulocytic ehrlichiosis: a case series from a medical center in New York State. *Ann Intern Med* 1996; 125: 904-8.
53 - Bakken JS, Krueth J, Wilson-Nordskog C, Tilden RL, Asanovich K, Dumler JS. Clinical and laboratory characteristics of human granulocytic ehrlichiosis. *JAMA* 1996; 275: 199-205.
54 - Horowitz HW, Marks SJ, Weintraub M, Dumler JS. Brachial plexopathy associated with human granulocytic ehrlichiosis. *Neurology* 1996; 46: 1026-9.
55 - Shea KW, Calio AJ, Klein NC, Cunha BA. *Ehrlichia equi* infection associated with rhabdomyolysis. *Clin Infect Dis* 1996; 22: 605.
56 - Wong S, Grady LJ. Ehrlichia infection as cause of severe respiratory distress. *N Eng J Med* 1996; 334: 273.
57 - Bakken JS, Erlemeyer AS, Kanoff RJ, Silvestrini TC, Goodwin DD, Dumler JS. Demyelinating polyneuropathy associated with human granulocytic ehrlichiosis. *Clin Infect Dis* 1998; 27: 1323-4.
58 - Jahangir A, Kolbert C, Edwards W, Mitchell P, Dumler JS, Persing DH. Fatal pancarditis associated with humam granulocytic ehrlichiosis in a 44-year-old man. *Clin Infect Dis* 1998; 27: 1424-7.
59 - Peters TR, Edwards KM, Standaert SM. Severe ehrlichiosis in an adolescent taking trimethroprim--sulfamethoxazole. *Pediatr Infect Dis J* 2000; 19: 170-1.
60 - Walker DH. Task Force on Consensus Approach for Ehrlichiosis. Diagnosing human ehrlichiosis: current status and recommendations. *ASM News* 2000; 66(5):287-90.
61 - Aguero-Rosenfeld ME, Kalantarpour F, Baluch M, Horowitz HW, Mckenna DF, Raffalli JT, et al. Serology of culture-confirmed case of human granulocytic ehrlichiosis. *J Clin Microbiol* 2000; 38: 635-8.
62 - Aguero-Rosenfeld ME. Diagnosis of HGE - state of art. Presentation summaries of the *IX International Conference on Lyme Borreliosis and Other Tick-borne Diseases.* New York; 18-22 Ago. 2002, p. 93-6.
63 - Bakken JS, Dumler JS. Ehrlichiosis. In: Cunha BA, edit. *Tick-borne Infectious Diseases.* New York: Marcel Dekker; 2000, p. 139-68.
64 - McQuiston JH, Paddock CD, Holman RC, Childs JE. The human ehrlichioses in the United States. *Emerg Infect Dis* 1999; 5: 635-42.
65 - Lotric-Furlan S, Petrovec M, Avsic-Zupanc T, Nicholson WL, Sumner JW, Childs JE, et al. Human ehrlichiosis in central Europe. *Wien Klin Wochenschr* 1998; 110: 894-7.
66 - Olano JP, Walker DH. Human ehrlichiosis. *Medical Clinics of North America* 2002; 86(2): 375-91.
67 - Goodman JL, Nelson C, Vitale B, Madigan JE, Dumler JS, Munderloh UG. Direct cultivation of the causative agent of human granulocytic ehrlichiosis. *N Engl J Med* 1996; 334: 209-215.
68 - Ravyn MD, Goodman JL, Kodner CB, Westad DK, Coleman LA, Engstrom SM, et al. Imunodiagnosis of human granulocytic ehrlichiosis by using culture-derived human isolates. *J Clin Microbiol* 1998; 36(6): 1480-8.
69 - Zhi N, Ohashi N, Rikihisa Y, Horowitz HW, Wormser GP, Hechemy K. Cloning and expression of the 44 kilodanton major outer membrane protein gene of the human granulocytic ehrlichiosis agent and application of the recombinant protein to serodiagnosis. J Clin Microbiol 1998; 36: 1666-73.
70 - Ijdo JW, Wu C, Magnarelli LA, Fikrig E. Serodiagnosis of human granulocytic ehrlichiosis by a recombinant HGE-44-based enzyme-linked immunos orbent assay. *J Clin Microbiol* 1999; 37(11): 3540-4.
71 - Unver A, Felek S, Paddock CD, Zhi N, Horowitz HW, Wormser GP, et al. Western blot análisis of sera reactive to human monotytic ehrlichiosis and human granulocytic ehrlichiosis agents. J Clin Microbiol 2001; 39(11): 3982-6.
72 - CDC – Centers for Diseases Control and Prevention. Case definitions for infectious conditions under public health surveillance. *MMWR Morb Mortal Wkly Rep* 1997; 46: 46-7.
73 - Nadelman RB, Horowitz HW, Hsieh T-C, Wu JM, Aguero-Rosenfeld ME, Schwartz IS, et al. Simultaneous human granulocytic ehrlichiosis and Lyme borreliosis. *N Eng J Med* 1997; 337: 27-30.
74 - Schaffner W, Standaert SM. Ehrlichiosis - in pursuit of an emerging infection. *N Eng J Med* 1996; 334: 262-3.
75 - Buitrago MI, Ijdo JW, Rinaudo P, Simon H, Copel J, Gadbaw J, et al. Human granulocytic ehrlichiosis during pregnancy treated successfully with rifampin. *Clin Infect Dis* 1998; 27: 213-5.
76 - DGS. Doenças de declaração obrigatória 1995-1999. Lisboa: Direcção de Serviços de Informação e Análises, Divisão de Epidemiologia, Direcção-Geral de Saúde; 2000, p. 53.
77 - David de Morais JA, Leitão AL, Páscoa B, Filipe AR, Núncio MS. Doença de Lyme A nossa experiência clínica na região do Alentejo. *Rev Port Doenças Infec* 1992; 15(4): 227-45.
78 - Núncio-Soares MS. Contribuição para o estudo de borrélias e borreliose de Lyme em Portugal. Tese doutoral. Lisboa: Faculdade de Ciências da Universidade de Lisboa; 2002.
79 - Mitchell PD, Reed KD, Hofkes JM. Immunoserologic evidence of coinfection with *Borrelia burgdorferi, Babesia microti* and Human Granulocytic Ehrlichia species in residents of Wisconsin and Minesota. *J Clin Microbiol* 1996; 34(3): 724-7.
80 - Magnarelli LA, Ijdo JW, Anderson JF, Padula SJ, Flavell RA, Fikrig E. Human exposure to a granulocytic ehrlichia and other tick-borne agents in Connecticut. *J Clin Microbiol* 1998; 36(10):2823-7.
81 - Bjoersdorff A, Brouqui P, Eliasson I, Massung RF, Wittesjo B, Berglund J. Serological evidence of ehrlichia infection in swedish Lyme borreliosis patients. *J Infect Dis* 1999; 31(1): 51-5.