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INSTITUT AGRONOMIQUE ET VETERINAIRE HASSAN II

ANAPLASMA SPP IN DOGS AND HUMANS IN MOROCCO

Sarah El Hamiani Khatat

Student number (UGhent): 01311633
Order number (IAV Hassan II): 2017/10/VETO

Supervisor (s): Prof. Dr. Sylvie Daminet, Prof. Dr. Hamid Sahibi

A dissertation submitted in the fulfillment of the requirements for the degree of Doctor of Philosophy (Ph.D.) in Veterinary Sciences of Ghent University (UGhent) and l'Institut Agronomique et Vétérinaire Hassan II (IAV Hassan II)

Academic year: 2016-217

Defended in May 22th, 2017 before the examination committee consisting of:

Mrs M. KACHANI	**Professor, Western University of Health Sciences, the USA**	**President**
Mrs M. RIYAD	**Professor, Hassan II University, Morocco**	**Recorder**
Mr L. DUCHATEAU	**Professor, Ghent University, Belgium**	**Recorder**
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Anaplasma spp. in dogs and humans in Morocco

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ABBREVIATIONS

ACVIM	American College of Veterinary Internal Medicine
ankA	Ankyrin A protein
AP-ha	*A. phagocytophilum* human pathogenic variant
AP-variant1	*A. phagocytophilum* variant 1
AP-variant2	*A. phagocytophilum* variant 2
AP-variant3	*A. phagocytophilum* variant 3
AP-variant4	*A. phagocytophilum* variant 4
CDC	Center of Disease Control and Prevention
CGA	Canine granulocytic anaplasmosis
DNA	deoxyribonucleic acid
drhm	Distantly related to human marker
EDTA	Ethylenediaminetetra-acetic acid.
ELISA	Enzyme-linked immunosorbant assay
G+C content	Guanine-cytosine content
HGA	Human granulocytic anaplasmosis
HGE	Human granulocytic ehrlichiosis
ICG	Immunochromatography
IFA	Immunofluorescence assay
ICCT	Infectious canine cyclic thrombocytopenia
Mb	Megabase
MMWR	Morbidity and Mortality Weekly Report
MODS	Multiple organ dysfunction syndrome
msp2/p44	Major surface protein 2/p44
msp4	Major surface protein 4
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
RT-PCR	Real-time polymerase chaine reaction
SIRS	Systemic inflammatory response syndrome
TBDs /TBPs	Tick-borne diseases/pathogens
TBF	Tick-borne fever
VBDs/VBPs	Vector-borne diseases/pathogens
VNRT	Variable number tandem repeat
WA	Washington canine *A. phagocytophimun*
WB	Western blot

CHAPTER I

GENERAL INTRODUCTION

This chapter is divided in five parts.

In the first part, a general introduction on the importance of vector-borne diseases worldwide is provided and factors that contributed to their expansion and increasing interest are explained. This chapter focusses on tick-borne diseases and more specifically on *Anaplasma phagocytophilum* and granulocytic anaplasmosis.

The second, third and fourth parts describe the main characteristics of *A. phagocytophilum* and *Anaplasma platys* including their classification, morphology and structure. An overview of the most important epidemiological features of both bacteria including the transmission modes, the reservoir host range, the life cycle, their zoonotic potential and an emphasis on the epidemiological roles of dogs in both infections are also exposed.

The fifth part summarizes the main studies about the distribution and prevalence of both *A. phagocytophilum* and *A. platys* worldwide and discusses the limitations of prevalence studies.

1. Vector-borne diseases gain interest

Vector-borne diseases (VBDs) are caused by various infectious agents including parasites, bacteria and viruses that are transmitted to a host through the bite of hematophagous arthropods. A wide variety of VBDs are zoonoses,[1] i.e., infections or infectious diseases transmissible under natural conditions from vertebrate animals to humans. Zoonoses comprise almost 60% of all known infectious diseases and 75% of emerging infectious agents are zoonotic.[2] VBDs impact human and animal health and the global economy, representing approximately 17% of the burden of all infectious diseases, causing one billion cases, over one million deaths and millions of dollars in losses to the livestock industry annually.[3,4] In addition, many people who survive infection are left permanently debilitated, disfigured, maimed, or blind. One sixth of the illness and disability suffered worldwide is due to VBDs, with more than half of the world's population currently estimated to be at risk of these diseases.[3] Their distribution is determined by a complex dynamic of environmental and social factors.[4] Although many VBDs affect mostly the least-developed countries such as malaria, dengue, schistosomiasis, leishmaniasis, Chagas disease, yellow fever, lymphatic filariasis and onchocerciasis, others are more prevalent in Europe and the USA such as Lyme disease and tick-borne encephalitis virus.[5] Among VBDs, canine VBDs have been of increasing interest the past decades due to the close relationship between dogs and humans. Indeed, dogs share the same environment as humans; hence they are exposed to similar vectors. In addition, dogs may play important epidemiological roles as competent reservoirs host of vecor-borne pathogens (VBPs), source of infection for vectors, mechanical transporters of infected vectors, or effective sentinels of regional infection risk for humans.[6-13]

Ticks display a worldwide distribution and are considered to transmit the widest number of pathogens when compared to other arthropod vectors, producing the highest number of human disease cases in some regions of the world.[13,14] Indeed, in North America and parts of Europe, Lyme disease transmitted by *Ixodes* spp ticks is the most important VBD and a main cause of human morbidity, surpassing any mosquito-borne disease. Lyme disease is responsible for more than 90% of all VBD cases in the USA and it may be responsible for disease in 255,000 persons annually worldwide, mostly in Europe and North America.[13,15,16] According to the USA Morbidity and Mortality Weekly Report (MMWR), a total of 484,352 cases were reported between 1992 and 2015, with a steady increase of 287% in the number of reported cases during this period.[17,18] In Europe and China there is an estimated average of 85,000 and 30,000 cases per year, respectively.[19,20] Tick-borne diseases (TBDs) are also responsible for several diseases in domestic animals causing serious illness, mortality and major depression in livestock production worldwide.[21-24] Unlike other human flying arthropod-borne diseases where infection can be independent of association with animals and humans are the main host, TBDs

are overwhelmingly zoonotic and humans are usually incidental hosts. Therefore, integration of veterinary and human reporting systems, surveillance in wildlife and tick populations, and combined teams of experts from several scientific disciplines such as entomology, epidemiology, medicine, public health and veterinary medicine are needed for the formulation of regulations and guidelines for the prevention of TBDs.[13]

Within the past decades, several VBDs have been considered to be emerging or re-emerging because they are newly recognized within an area or because of an increase in their incidence or expansion of their geographical distribution or host or vector range.[2,5,7] Many VBDs have been reported in previously not affected areas such as babesiosis in northern Germany, Belgium, Poland and the Netherlands. *Anaplasma platys* seems to be more frequently diagnosed in Europe, *Candidatus* Neoehrlichia mikurensis seems to extend its distribution worldwide. Canine monocytic ehrlichiosis, granulocytic anaplasmosis, tick-borne encephalitis virus and *D. immitis* are reported to extend to northern Europe. Similarly, leishmaniasis is spreading to Northern Europe and Northern America.[25-31] Multiple factors are supposed to play a crucial role in arthropod-vectors expansion mainly increased animal travelling and migration, climatic changes with a global warming, landscape rehabilitation and management with increased urbanization, development of large suburban areas with private gardens, creation of artificial lakes, forests modification, increased popularity of open-air activities, changes in wildlife fauna, loss of biodiversity, decreased host population densities, and residential growth expanding into rural geographic areas. All these conditions affect the ecology and epidemiology of infectious diseases, enable the circulation, multiplication and spread of both vectors and pathogens into formerly unaffected areas, promote the creation of niches for vectors and their capacity to newly acquire pathogens, impact wildlife populations that serve as reservoirs and the dynamic of transmission amongst natural reservoirs, increase the risk for the host to enter in contact with vectors and impact the likelihood of animal–human transmission.[1,5,30,32-34] Therefore, although traditionally regarded as a problem for countries in tropical settings, VBDs pose an increasingly wider threat to global public health, both in terms of the number of people affected and their geographical spread.[5] Beside these environmental changes leading to increased hazard exposure to VBPs, increased clinician awareness, new diagnostic tools, improved surveillance and increased reporting and communication of these diseases in several countries can also explain the increased incidence.[1,21,30,34] Advances in molecular biology also allow the discovery of new species, strains or genetic variants and extend the list of VBPs able to infect either animals or humans or both.[13,35,36] Finally, VBP spectrum seems to expand, and some pathogens traditionally associated with domestic animal infections may also potentially emerge as human pathogens[13] such as *Ehrlichia canis*[37,38] and *Anaplasma platys*.[39-41]

Anaplasma phagocytophilum, the agent of granulocytic anaplasmosis, is considered as an emerging zoonotic tick-borne pathogen.[42] Indeed, the environment suitability of its main vector seems to increase in Canada[43] where human granulocytic anaplasmosis (HGA) is of growing concern for public health due to the recent establishment of *Ixodes scapularis* in southeastern and south central regions.[44] In the USA, human and canine infections with *A. phagocytophilum* have been reported in the Pacific northwest, the upper Midwest, and the northeastern and mid-Atlantic USA, and most cases occur in Minnesota, Wisconsin, New York state, New Jersey, and Connecticut, suggesting that expansion from the USA may further drive the emergence of this tick-borne disease in Canada.[32] Possible implication of migratory birds in the expansion of *I. scapularis* ticks in Canada (especially in northern provinces) has also been suggested.[45] In the USA, HGA is a nationally notifiable disease and both canine and human exposure to *A. phagocytophilum* has progressively increased from 2008 to 2010.[46-49] Data from the USA Center for Disease Control and Prevention (CDC) and MMWR reported 10,670 human cases between 2010 and 2013, and an 8-fold increase in reported cases between 2000 and 2013.[18,50,51] In Europe, high prevalence rates of *A. phagocytophilum* were observed in both Ixodid ticks and wild animals.[52-55] *Ixodes ricinus*, the main vector of this bacterium in Europe,[23] has expanded its territories over the past few years in European countries due to several factors including climatic and ecological modifications and also probably because of a low host specificity and tolerance to various environments.[56-59] Serological evidence of *A. phagocytophilum* infection and granulocytic anaplasmosis have been reported in several European countries in both dogs[60-66] and humans.[67-75] Currently, HGA is considered the third most important VBD in both the USA and Europe, and is also increasingly diagnosed in some Asian countries.[77,76] In China, *A. phagocyophilum* exposure among high-risk populations seems to have rapidly increased and reported cases showed a higher severity and mortality than in the USA and Europe.[78] Despite the increased reporting of this infection, it is still unrecognized and underdiagnosed.[79] Moreover, its occurrence is unknown in large parts of the world including Africa, Oceania, South America and many Asian countries.[80,81] In North Africa, ticks are abundant and might represent potential hazard for animal and human public health. Evidence of Anaplasmataceae species infection in various tick species have already been reported.[82,83] However, only a few epidemiological data are available on *A. phagocytophilum* in this continent mostly on ticks[84,85] and domestic animals (ruminants, horses and dogs)[86-88] but studies on human exposure are still missing.

2. Classification and morphology of *Anaplasma phagocytophilum* and *Anaplasma platys*

2.1 Classification of Anaplasma phagocytophilum and Anaplasma platys

Anaplasma phagocytophilum and *A. platys* are α-proteobacteria belonging to the family of Anaplasmataceae in the order of Rickettsiales. The order Rickettsiales is divided in two families: Anaplasmataceae and Rickettsiaceae (Figure 1). The family Anaplasmataceae includes agents of *Ehrlichia*, *Anaplasma*, *Wolbachia*, *Neorickettsia*, *Cowdria* genera and provisionally the genus *Aegyptianella*. Except for *Wolbachia*, the family of Anaplasmataceae includes obligate intracellular arthropod-borne bacteria that infect mature and immature hematopoietic cells and develop within intracytoplasmic vacuoles.[89] They are responsible of endemic and emerging diseases of major relevance in both veterinary and human medicine with important economic and public health outcomes (Table 1).[90] Seven *Anaplasmataceae* organisms are able to infect humans namely, *Ehrlichia chaffeensis*, *E. ewingii*, *E. canis*, *E. ruminatum*, *A. phagocytophilum*, *A. platys*, *Neorickettsia sennetsu* and ‘‘*Candidatus* Neoehrlichia mikurensis’’ (Table 1) but only the former three species are sufficiently investigated because they are responsible of the majority for human ehrlichiosis and anaplasmosis cases.[37,91-93]

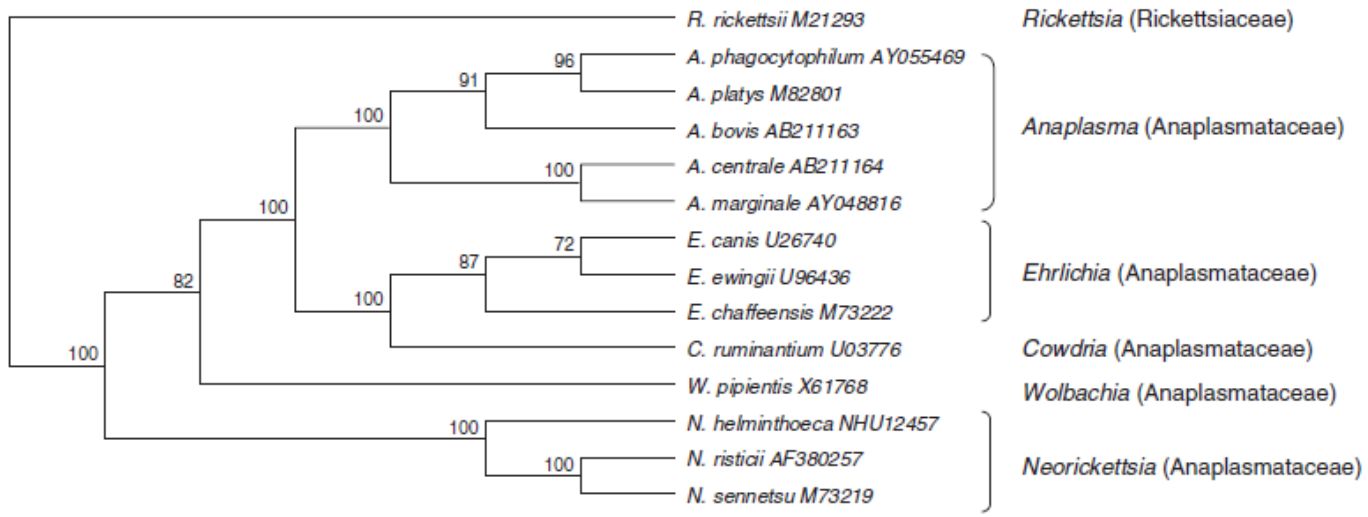

Figure 1. Phylogenetic tree of the order Rickettsiales on the basis of the *16S rRNA* gene sequencing, showing relationship between the belonging agents (species and family name on the right in parentheses). Bootstrap percentages are noted at the nodes of the tree. Bar, 10 substitutions per 100 nucleotides.[10]

Table 1. Rickettsial agents belonging to the family Anaplasmataceae infecting companion animals and human. Adapted from 90,94

Agents	Primary vector	Distribution	Host cells	Susceptible species and disease
Anaplasma phagocytophilum	*Ixodes persulcatus* complex	Worldwide	Neutrophils Eosinophils	Granulocytic anaplasmosis, tick-borne fever, tick-borne pasture Humans, dogs, cats, horses, ruminants
Anaplasma platys	*Rhipicephalus sanguineus?* *Dermacentor auratus?*	Worldwide	Platelets	Dogs: Infectious canine cyclic thrombocytopenia Cats: questionable
Ehrlichia canis	*Rhipicephalus sanguineus* *Dermacentor variabilis*	Worldwide	Monocytes Macrophage Lymphocytes	Dogs: canine monocytic ehrlichiosis Cats: fever, lethargy, anorexia Human : infection identified in Venezuela
Ehrlichia chaffeensis	*Amblyomma americanum*	Southern USA Eastern USA California	Monocytes Macrophages Lymphocytes	Human : human monocytic ehrlichiosis Dogs: mild/subclinical unless present in co-infection
Ehrlichia ewingii	*Amblyomma americanum*	Southern USA Eastern USA	Neutrophils Eosinophils	Human : human granulocytic ehrlichiosis, uncommon Dogs: granulocytic ehrlichiosis
Ehrlichia ruminantium	*Amblyomma* spp.	Africa Caribbean	Endothelium Monocytes Nacrophages Neutrophils	Ruminants : heartwater Dogs: subclinical/rare
Neorickettsia risticii	*Acanthatrium oregonense,* caddisflies, aquatic insects	North America	Monocytes macrophages Enterocytes	Dogs: lethargy, fever, vomiting, arthritis, thrombocytopenia Cats: experimental infection
Neorickettsia helminthoeca	Trematodes: *Nanophyetus salmincola*	Northwest USA British Columbia Brazil	Monocytes Macrophages Enterocytes	Dogs: fever, anorexia, diarrhea, vomiting, lymphadenopathy

Anaplasma phagocytophilum is known as a veterinary pathogen since the discovery of tick-borne fever (TBF) in Scotland in 1932.[95] The taxonomic position and the name of the bacterium changed several times being successively named *Rickettsia phagocytophila*,[96] *Cytoecetes phagocytophila*[97] and *Ehrlichia phagocytophila*.[98] *A. platys*, the agent of Infectious canine cyclic thrombocytopenia, was first reported in the USA in 1978 and was first named as *Ehrlichia platys.*[99] Phylogenetic molecular analysis based on the *16S rRNA*, *groESL* and surface protein genes sequencing in addition to morphologic and phenotypic characteristics have led to the reorganization of the Anaplasmataceae family and the reclassification of some agents. Consequently, *E. platys* was renamed as *Anaplasma platys*. Similarly, the name *A. phagocytophilum* was given in 2001 to three previously distinct agents, i.e., the agent that causes equine granulocytic anaplasmosis (*Ehrlichia equi*), the agent that causes tick-borne fever or

pasture fever in sheep and cattle, respectively (*Ehrlichia phagocytophila*) and the agent that causes HGA.[89,100] The renaming of these three agents as *A. phagcytophilum* has been controversial because of differences in their host tropism and cell target from other *Anaplasma* species such as *Anaplasma marginale*.[101] Additionally, although these three agents share genetic, antigenic and biological characteristics,[89] they are considered phenotypic variants due to differences in their distribution, prevalence, virulence and target host species.[102,103]

2.2 Morphology and structure of Anaplasma phagocytophilum and Anaplasma platys

Anaplasma phagocytophilum and *A. platys* are gram-negative, non-motile pleomorphic bacteria that mostly display a coccoid to ellipsoid shape. Their sizes vary from 0.2 to 2.0 μm and 0.3 to 1.2 μm in diameter, respectively. Like the other members of the *Anaplasma* genus, these bacteria are obligate aerobe that lack glycolytic pathway. Their membrane is rippled, thin and lacks the peptidoglycan layer and lipopolysacharrides of the cell wall. These two features make them sensitive to mechanical stress including freezing, thawing, sonication and osmolarity changes.[99,104-107] Both bacteria infect peripheral blood cells derived from bone marrow precursor with *A. phagocytophilum* infecting preferentially neutrophils but also occasionally eosinophils whereas *A. platys* parasitizes circulating platelets.[99,106,108] *A. platys* is also able to infect megakaryocytes and promegacaryocytes of the bone marrow in naturally infected dogs.[109] These organisms develop within intracytoplasmic inclusions of varying size (from 1.5 to 6 mm in diameter) derived from the host cell membrane. These vacuoles are endosomes where the bacteria find nutrient and multiply by binary fission.[79,81,110-112] The vacuoles can contain two distinct ultrastructural forms characterized by their DNA organization, i.e., a small dense core with condensed protoplasm also called 'elementary body' (0.2 to 0.4 μm) or a large reticulated form named 'reticulate body' (0.8 to 2.0 μm).[79,81,90,110,111] Both forms can replicate by binary fission producing 1 to 20 organisms forming a "morula" (from Latin *morum*: "mulberry") (Figure 2).

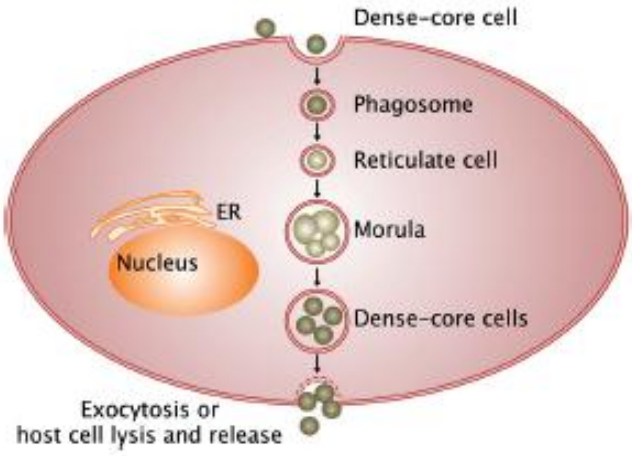

Figure 2. Intracellular development of Anaplasmataceae pathogens.[90]

Morulae appear as basophilic intracellular inclusions of varying size from 1.5 to 2.5 μm; but can be as large as 6 μm.[81,99, 106,108,110] Other authors consider that only the reticulated forms (vegetative form) multiply by binary fission until forming morulae, and then turns into the dense cored cells (infectious form), which are released and bind to host's cells target.[10,90,113] Morulae of *A. platys* (Figure 3A) and *A. phagocytophilum* (Figure 3B) are detectable in peripheral blood smear 9 to 17 days and 4 to 14 day after experimental inoculation, respectively.[99,114-117]

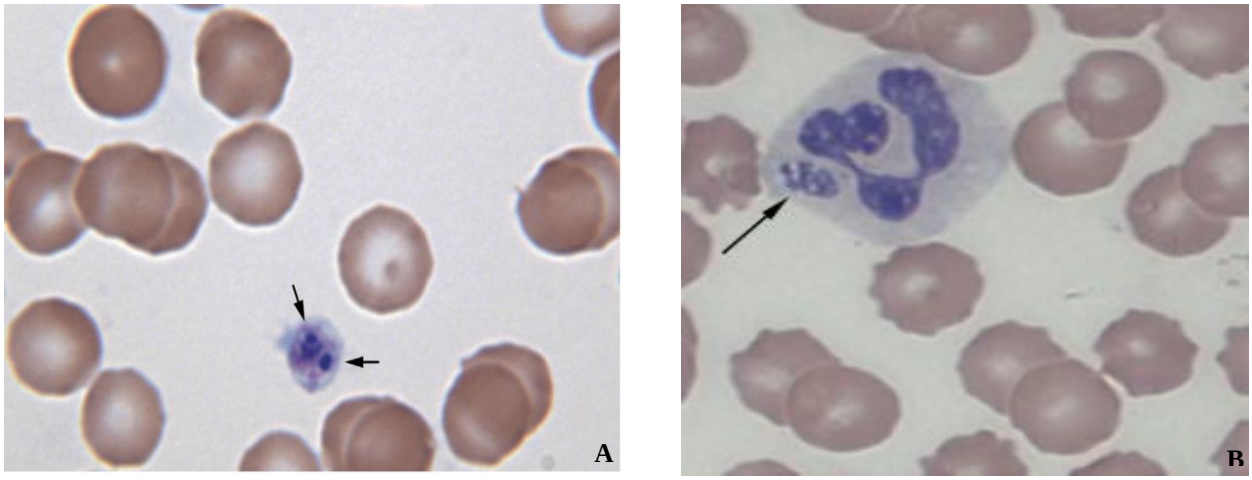

Figure 3. ***Anaplasma platys*** **(A) and** ***Anaplasma phagocytophilum*** **(B) intracytoplasmic inclusions (morulae)** within a canine peripheral blood platelet after experimental infection and a canine neutrophil, respectively. Morulae appear as purple stained bodies within the platelet cytoplasm (arrow).[10,117]

The only bacteria, other than *A. phagocytophilum*, known to survive and multiply in neutrophils are *E. ruminantium, E. ewingii*, and *Chlamydophila pneumonia.*[79] By light microscopy, morulae of *Ehrlichia ewingii* are identical to those of *A. phagocytophilum*. This can lead to misdiagnosis in the regions where both pathogens are present if only blood smear examination are performed.[10,81]

3. *Anaplasma phagocytophilum*

3.1 Genome

The *A. phagocytophilum* genome is composed by a single circular double-stranded chromosome. The complete genomic sequence is estimated at 1.47 megabases (Mb) that contains 41.63% of G+C content, 1,369 open reading frames and 458 hypothetical proteins but lackes any associated plasmids.[118] The complete genomic sequence of the bacterium has been submitted to GenBank in 2006 (NC007797) and comprises between 1,140 and 1,411 genes including protein coding sequences, rRNA, tRNA, and pseudogenes.[118,119] This genome also contains many repeated sequences that are associated with important functions such as the expansion of outer membrane protein of the msp2/p44 family, type IV secretion system, vitamin/cofactor biosynthesis and many variable number tandem repeat (VNTR) sequences.[118-121] The genome contains 113 "functional pseudogenes" *msp2*/*p44* that encodes for the major surface antigen. These hypervariable pseudogene are recombined into a single expression site which enables the bacterium to serially express variable antigens and to escape from host immunity.[118,122,123] Outer membrane proteins play several essential roles in the adaptation of the bacterium to variable environments and host niches, the transport of nutrients and molecules acting in the host-interaction, antimicrobial resistance, response to osmotic stress.[124,125] and inducing neutralizing antibodies against homologous strains of *A. phagocytophilum.*[126,127] Type IV secretion system has a crucial role in the pathogenesis of the disease.[128] Some genes may also contribute to the resistance of the bacterium in diverse environments.[118]

Despite the apparently genome simplicity of *A. phagocytophilum*, this bacterium exhibits an extensive genomic diversity.[120,129,130] More than 500 partial pseudogene sequences derived from human, tick and animal strains from several USA, European and Asian regions are available in GenBank.[120] Moreover, twenty complete *A. phagocytophilum* genomes have been sequenced including sixteen American strains and four European strains. However, only a few genomes per host species are available, except for humans, which might underestimate the true strain diversity.[56,118-120]

3.2 Genetic variability

The genetic variability has been suggested as an explanation to the ecological complexity, host tropism diversity and the observed differences in incidence, clinical severity, and disease manifestation between geographic regions.[118,131-133] Clinical cases of granulocytic anaplasmosis in ruminants have exclusively been reported in Europe while only a few human cases have been described in this continent. In contrast, USA strains do not cause disease in domestic ruminants but a higher human incidence rate and severity of the disease were reported.[56,120] In western states of the USA, the discordance between the distribution of clinical cases in humans, dogs and horses and the infection in the reservoir hosts suggests that multiple strains are circulating.[134] Genetic variants from Rhode Island and Connecticut could interfere with the transmission and maintenance of strains causing disease in humans. This presumed host competition between different variants could explain the lower incidence of human cases in some areas.[130,135,136] Variants causing the disease in sheep and cattle failed to induce the disease in horses. Conversely, isolates from horses induced seroconversion but not clinical signs in lambs and cattle when inoculated.[137-139]

Genetic variability has been demonstrated first by the sequencing of the *16S rRNA* gene.[140-142] The TBF variant differs from the human variant in three positions of the *16S rRNA* gene.[143] The sequencing of the 5' region of this gene enabled the identification fifteen *A. phagocytohilum* variants, respectively.[113] In the USA, several variants have been identified based on the sequencing of the *16S rRNA* and the only pathogenic variant to humans (Ap-ha) is also able to induce the disease in dogs, horses and mice but not in cattle. Another strain (Ap-variant 1) circulating in deer (*Odocoileus* spp), is genetically distant from the Ap-ha strain and infects only deer, goats (*Capra aegagrus hircus*), and tick-origin cell lines, while experimental infection of mice was not successful.[129-131,135,144] In Europe, other variants have been identified in humans and the Ap-ha variant has also been detected in wild ruminants.[56,118,141] Strains infecting domestic ruminants in Europe and white-tailed deer in the USA (Ap-variant 1) seem to genetically differ from those infecting humans, horses and dogs.[132,145] The Ap-ha and Ap-variant 1 can coexist in the same geographic area, could be transmitted by the same vectors, and seem to segregate only according to their host tropism.[146] Similarly, multiple *16S rRNA* variants can coexist in a single infection and several phenotypically untyped variants have been reported in Europe and the USA.[147,148] In Washington, five different *16S rRNA* variants (named WA1 to 5) that differed at four nucleotide positions were identified from dogs displaying clinical signs consistent with granulocytic anaplasmosis. All WA variants were distinct from those identified in sheep in Norway and llama-associated ticks but one was identical to equine and human variants.[136] In another European study, seven different *16S rRNA* variants were identified from dogs, with the two most common variants

showing statistically significant differences in the frequency of clinical signs and hematological abnormalities, suggesting possible differences in strain pathogenicity.[133]

The *16S rRNA* gene was considered too conserved for use in phylogenetic analysis between different strains of *A. phagocytophilum*. It had poor resolution and failed to discriminate between ecotypes circulating in wild ruminants compared to other animals. Furthermore, the *16S rRNA* sequence analysis could not categorize human-infective isolates in order to detect virulent strains and was unable to distinguish variants according to their geographic origin.[52,131,140,141] Consequently, other genes have been proposed to study the genetic variability of *A. phagocytophilum* including *msp4*, *ankA*, *groEL* operon, *msp2/p44* genes.[52,122,142,149,150] The genes encoding outer membrane proteins of the *OMP-1/msp2/p44* protein superfamily are involved in the interactions with the hosts and vectors. The high variability of *msp2/p44* is associated with multiple antigenic variations that arise during the *A. phagocytophilum* reproduction in mammals and ticks and facilitate bacterial survival in diverse hosts and persistence in vertebrate reservoir hosts.[107,113,118,151] A comparison of the *msp2/p44* sequences of ruminant and tick isolates from Europe and the USA have demonstrated that most of the sequences displayed only moderate identity to one another, and any distinct clustering of sequences from individual isolates, from different countries, or different host species was absent. Therefore, it has been hypothesized that the sequences of *msp2/p44* gene in similarity groups may provide an index of adaptation of *A. phagocytophilum* strains to specific vectors or reservoir hosts in different geographical locations.[122,152-154] In contrast, the *msp4* gene sequences are genetically stable during the multiplication in hosts' cells; thus, it is preferable for phylogenetic analysis. The analyzed strains of *A. phagocytophilum* showed a high degree of identity in the *msp4* locus.[141,155] The *ankA* gene encodes an ankyrin repeat protein involved in host cell transcription regulation named the ankyrin repeat-containing protein (*ankA*) (153-160 kDa).[56,113] This gene is suspected to play a fundamental role in the pathogenesis by interfering in the transcription of some genes.[79,156] The *ankA* gene enables the discrimination between animal host tropism only[52] and some authors consider that it could not display the required level of discrimination for epidemiological studies.[56] The gene sequences seem to vary according to the geographic location and show a relative conservation among North American strains as opposed to European isolates[79] except for human European strains that seem identical.[150] European variants and American human variants were segregated in separate subgroups. Sequences of this gene were found to divide in distinct variant clusters associated with animal host tropism. Isolates from humans, horses, dogs and cats were found exclusively in the same cluster, which also included several variants from domestic and wild ruminants. Another cluster was composed of variants from wild ruminants (roe deer and red deer) while the third one included variants isolated from both wild (red and roe deer) and domestic ruminants (cattle and sheep). Two other clusters included exclusively variants isolated from

roe deer and rodents, respectively.[52,56,157] Another study found four distinct animal hosts tropism ecotypes with different enzootic cycles based on the sequencing of the *groEL* heat-shock protein gene.[142] The *groESL* heat shock operon has an intermediate genetic variability and is expected to act as a marker for demographic analysis. Hence, it could more clearly discriminate between *A. phagocytophilum* isolates from different origin and further between isolates of different pathogenicity than the *16S rRNA* gene.[56,142,158] The first *groEL* cluster contained all human isolates and variants from wild (hedgehogs, mouflons, red deer) and domestic animals (cattle, dogs, horses and sheep). The second cluster included wild and domestic ruminant isolates (roe deer, red deer and sheep) and rodent variants. The third and the fourth clusters grouped exclusively isolates from rodents and birds, respectively.[142] Different gene sequencing revealed similarities between human and canine isolates, suggesting that dogs and humans may be infected by the same strains in Europe and the USA.[120,150,159-163]

All previous single gene based sequencing methods enabled the identification of geographic and/or host tropism clusters but failed to categorize human-infective isolates in order to detect virulent strains and had some contradictory results depending on the loci used. More recently, other methods such as multilocus strategy, whole genome sequencing or other locus targets[56,113,162,163] were proposed to help solve these problems. A gene named *drhm* (for 'distantly related to human marker') was suggested to be a potential valuable marker of human strain virulence because it was identified in several strains including the USA Ap-variant 1 (ruminant), MRK (horse) and the European sheep variant but deleted in strains infecting humans and dogs in the USA.[162] Despite the worldwide genomic diversity, human-infective strains seem to represent a conserved subset. Indeed, the homology between human-origin strains in the USA, Europe and Asia suggests that humans may not be susceptible to many of the circulating wildlife strains and that their susceptibility may be conditioned by selection pressures in small mammal reservoir hosts that cause evolution of novel strains able to invade and survive in humans.[120]

3.3 Vector

Anaplasma phagocytophilum is commonly described as a TBD because most contaminations of people and animals occur after tick bites[28] especially when they come in contact with the vector in reservoir hosts habitat.[164] *Anaplasma phagocytophilum* is transmitted mostly by hard ticks of *Ixodes persulcatus* or *I. ricinus* complex. The genus *Ixodes* includes approximately 245 species among them 14 belong to the *I. ricinus* complex. This complex contains four tick species that are involved in the transmission of the majority of *Ixodes*-vectored human diseases, i.e., *I. scapularis*, *I. pacificus*, *I. ricinus* and *I. persulcatus*.[23,80,89] Species of this complex are widely distributed throughout the world and are commonly found in the northern hemisphere (Figure 4). Their occurrence within a territory depends on climatic conditions (between 10 and 30°c, and >80% relative humidity) and the availability of feeding hosts.[8,23]

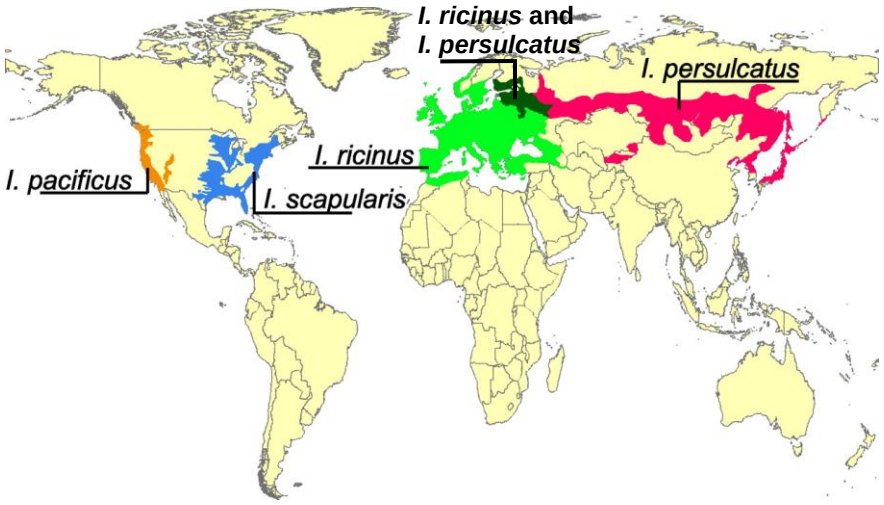

Figure 4. Worldwide geographic distribution of *Ixodes* spp. ticks, vectors of *Anaplasma phagocytophilum*. Adapted from 80

In the USA, several ixodid ticks are competent vectors of *A. phagocytophilum*, depending on the geographic location (Figure 4). The main vector in the humid forests of the upper Midwestern, north central and northeastern regions is *Ixodes scapularis* (Figure 5) whereas *Ixodes pacificus* (Figure 5) is located in shrub forests and deserts of the western USA.[165-168] Surveys from Canada suggest that *I. scapularis* ticks are also the most important vectors of *A. phagocytophilum* in this country.[169,170] The activity of *I. scapularis* varies during the year according to the life stage and the geographic localization. In the North of the USA, adult ticks are active from early spring to summer and in winter, nymphs are active during spring and summer whereas larvae activity extends from summer to fall. In the South, all stages are active from the end of fall until the end of spring.[1,165] The prevalence of *A. phagocytophilum* DNA among *I. scapularis* and *I. pacificus* ticks range from less than 1% up to 50% and 10%,

respectively.[171-174] Other tick species have been reported to be infected by *A. phagocytophilum* such as *Ampbyomma americanum* and *Dermacentor* spp., and *D. albipictus, I. spinipalpis* and *I. dentatus* are recognized as competent vectors.[175-179] In central and southern America, very few studies are published on the prevalence of *A. phagocytophilum* among ticks. However, among the three available studies, none have detected the DNA of this bacterium in *Ixodes* spp. ticks. In contrast, its DNA has been amplified from *Rhipicephalus sanguineus*, *Amblyomma cajennense*, *A. dissimile*, *A. maculatum*, *Dermacentor variabilis*.[180,181,182] *Amblyomma* spp. and *D. variabilis* were positively correlated with *A. phagocytophilum* infection in Brazil and Mexico.[180,182]

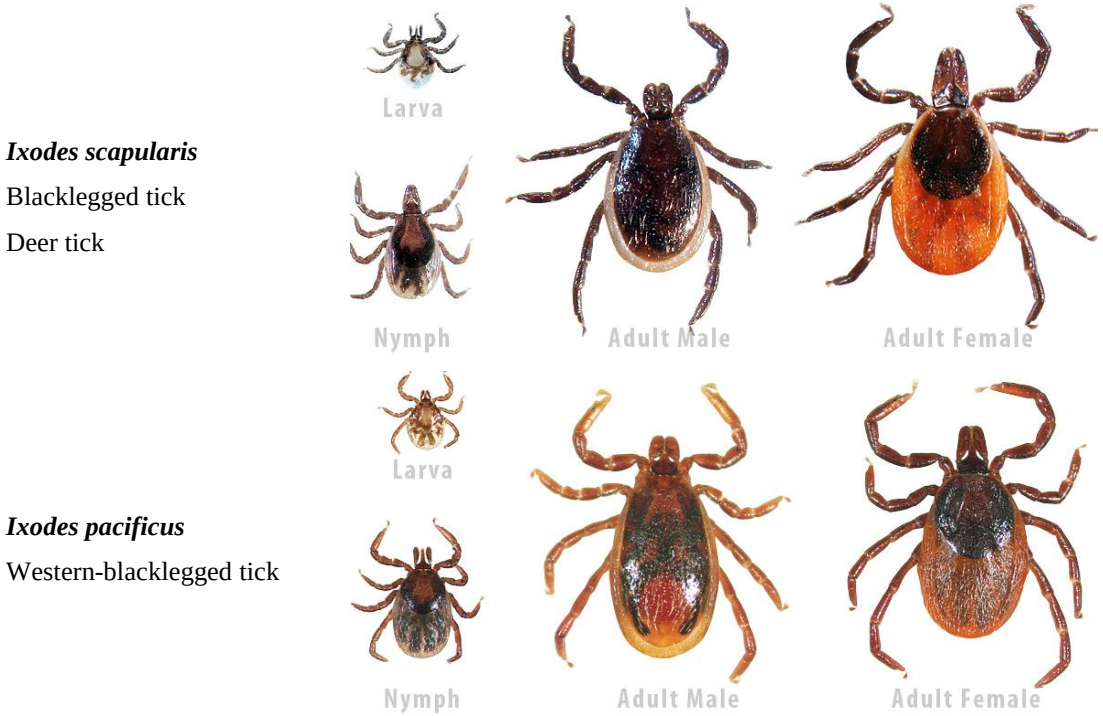

Figure 5. *Ixodes scapularis* and *Ixodes pacificus* ticks stages (TickEncounter Resource Center of the University of Rhode Island).

In Europe, the most common vector is *I. ricinus* (Figure 6),[23] which is widely distributed from Western Europe to central Asia (Figure 4). This tick lives mostly in humid wooded habitats and pastures and is rarely encountered in the Mediterranean region or in mixed or deciduous forests except at high altitudes.[28] It is active mostly in spring, from April to June.[42] The prevalence of *A. phagocytophilum* DNA among *I. ricinus* ticks in Europe range from less than 1% to 76.7%,[53,183] and variation according to the stage of development and between countries are reported to occur.[23,54] Other *Ixodes* spp. ticks seem to be involved in epidemiological cycles distinct from those involving *I. ricinus* including *I. trianguliceps, I. hexagonus* and *I. ventalloi*.[26,141,184-186] In addition, the DNA of this bacterium has been detected in several other tick species in Europe including *Dermacentor reticulatus* and *Hyalomma concinna*.[149,187,188] *Rhipicephalus* species were also infected with *A. phagocytophilum* and

could act as competent vectors in the Mediterranean area.[121,189-193] *Ixodes persulcatus* (Figure 7) is another competent vector of *A. phagocytophilum* in Eastern Europe and Asia (Figure 4), with prevalence rates reported to be up to 16.7% and 21.6%, respectively.[194,195]

Figure 6. ***Ixodes ricinus*** **tick developmental stages (sheep or castor bean tick)**.[196]

Although *I. persulcatus* is considered the primary vector in Asia, *A. phagocytophilum* DNA has been detected in several other tick species including *Ixodes nipponensis, I. ovatus, Rhipicephalus turanicus, R. microplus, R. sanguineus, Hyalomma marginatum, Boophilus kohlsi, Dermacentor silvarum* and several *Haemaphysalis* species.[190,197-201] Molecular investigations indicated that *I. ovatus, D. silvarum, H. concinna, H. longicornis, R. microplus, R. sanguineus* and *D. nuttalli* might be involved in the transmission *A. phagocytophilum* in China.[201-204]

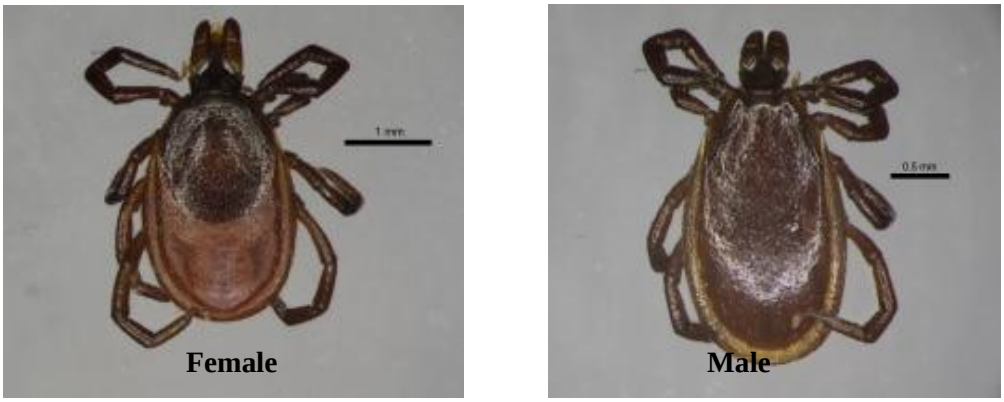

Figure 7. ***Ixodes persulcatus*** **tick** (Online photographic guide to ticks, Bristol University tick ID).

In North Africa, only a few studies have investigated the prevalence of *A. phagocytophilum* DNA among ticks. A survey from Morocco and Tunisia detected *A. phagocytophilum* DNA in 1% and 3% of *I. ricinus* and *Hyalomma detritum*, respectively.[82] Although *I. ricinus* has been suggested to be the main vector of *A. phagocytophilum* in this part of the world as well, some reports detected its DNA in several other tick species.[85] Indeed, two studies showed a prevalence of 13.7% and 2.3% in *R. sanguineus* (Figure 8) collected from free-roaming dogs in Egypt and *H. marginatum* collected from horses in Tunisia, respectively.[84,85] Additionally, *Hyalomma dromedarii*, *H. excavatum* and *H. impeltatum* ticks have been collected from dromedaries with positive antibody titers to *A. phagocytophilum* in Tunisia.[87] Therefore, *A. phagocytophilum* is likely to circulate in a wide variety of ticks feeding on a wide range of hosts; however whether all these ticks are involved in the transmission to hosts or not is still unestablished.[85]

Figure 8. ***Rhipicephalus sanguineus*** **ticks or brown dog ticks** (TickEncounter Resource Center of the University of Rhode Island).

3.4 Reservoir hosts

A reservoir host is defined as a biotic or abiotic environment that enables a pathogen to persist in a sustainable manner. As *A. phagocytophilum* is an obligate intracellular bacterium and not transovarialy transmission in *Ixodes* spp. ticks, its reservoirs should be animal hosts permitting its survival, particularly outside the activity period of its vectors. Although a wide range of domestic and wild animal species can be infected by the bacterium, hosts might fulfill several characteristics to be considered as reservoir hosts. Indeed, a host reservoir most be fed on by an infected vector tick at least occasionally, take up a critical number of the infectious agent during the bite by an infected tick, allow the pathogen to multiply and survive for a period in at least some parts of his body, and might allow the pathogen to find its way into other feeding ticks. Therefore, the detection of pathogens or their DNA in vertebrate hosts is not enough to consider them as reservoir hosts. If these hosts display also physiological and behavioral characteristics enabling the multiplication and transmission to the vector, they can be considered as candidate reservoir hosts. Otherwise, these animals can act as simple carrier or dead end hosts.[33,205,206]

Wild mammals are considered to be the main reservoir host of *A. phagocytophilum*. Wild cervids are the most common reservoir hosts because they develop a persistent subclinical infection[54] with white-tailed deer (*Odocoileus virginianus*) and roe deer (*Capreolus capreolus*) the main feeding hosts for ticks in Eastern USA and Europe, respectively.[54,57] Small mammals are also major feeding hosts for ticks.[207] The host reservoir range of *A. phagocytophilum* seems to differ according to the geographic localization.[23] In Europe, prevalence rates of *A. phagocytophilum* in wild ruminants range from 10% to more than 90%, with highest prevalence rates recorded for roe deer.[52,55,208,209] *Anaplasma phagocytophilum* is highly prevalent in other wild ruminant species that may act as efficient reservoir hosts in Europe including red deer (*Cervus elaphus*), feral goats (*Capra hircus*), fallow deer (*Dama dama)*, sika deer (*Cervus nippon*), moose (*Alces alces*), elks (*Alces alces*), alpine ibex (*Capra ibex*) and chamois (*Rupicapra rupicapra*).[24,56,210,211] Similarly, several small mammal species were found to be infected with *A. phagocytophilum* including bank vole (*Clethrionomys glareolus*), wood mouse (*Apodemus sylvaticus*), yellow-necked mouse (*Apodemus flavicollis*), common shrew (*Sorex araneus*) and European hedgehog (*Erinaceus europaeus*) with prevalence rates up to 85%.[186,212-215] However, even though *A. phagocytophilum* has been detected in a wide variety of wild animal species in Europe, reservoir hosts for the human pathogenic strain are still unknown.[150,216] Indeed, the reservoir competence of rodents is not established and cervids were reported to mainly disseminate variants that have not been isolated in humans, dogs, horses or domestic ruminants.[52,121,158,216-218] In addition, the phylogenetic analysis based on several loci (*groEL*, *msp4* and *ankA*) revealed that rodent

strains are clustered in different groups than *A. phagocytophilum* isolates from other animal species, making these rodent strains unlikely to circulate in *A. phagocytophilum* epidemiological cycles involving other mammals. Furthermore, according to recent studies, rodents could be reservoir hosts in an independent epidemiological cycle, involving only rodents as mammalian hosts.[142,157, 219]

The main reservoir hosts in the USA, with variations according to the region, are white-footed mouse (*Peromyscus leucopus*), white-tailed deer (*Odocoileus virginianus*), grey squirrels (*Sciurus carolinensis*), Eastern chipmunks (*Tamias striatus*), dusky-footed wood rats (*Neotoma fuscipes)* and southern red-backed voles (*Myodes gapperi*).[23,35,54,136] In the eastern USA, the white-tailed deer is the principal reservoir host of the *A. phagocytophilum* AP-variant 1 with reported prevalence rates up to 46.6%.[132,220] In contrast, rodents are considered the most important reservoir hosts of the bacterium in the northeastern, the upper Midwestern and the western coast of the USA. Both white-footed mouse (*Peromyscus leucopus*) and eastern chipmunks (*Tamias striatus*) were found to be the main reservoir hosts for the Ap-ha in Northeastern USA.[221] Other rodents such as southern red-backed vole (*Clethrionomys gapperi*) are considered competent reservoir hosts for *A. phagocytophilum*.[130,222,223] In the western states of the USA, among the most frequently infected small mammals species are dusky-footed woodrat (*Neotoma fuscipes*), western gray squirrel (*Sciurus griseus*), Douglas squirrel (*Tamiasciuris douglasii*), gray squirrel (*Sciurus carolinensis*), deer mouse (*Peromyscus maniculatus*) and red wood chipmunk (*Tamias ochrogenys*). DNA of the bacterium has been detected in several rodent species with prevalences ranging from 1.8% to 88.4%.[54,175,220,224-226] However, there is an important spatial discrepancy between human, canine and equine clinical disease in the western USA and infection in the supposed reservoir hosts, suggesting that multiple distinct *A. phagocytophilum* strains could circulate in the western USA ecosystems.[134]

In Asia, no information is available on the reservoir host's competence of wild animals for *A. phagocytophilum*.[199] Only a few studies have been carried on wild ruminants and *A. phagocytophilum* has been detected in sika deer and Korean water deer (*Hydropotes inermis*) with prevalence rates up to 46% and 63.6%, respectively.[227-230] Small mammals such as wood mouse (*Apodemus sylvaticus*), Korean field mouse (*Apodemus peninsulae*) and black striped field mouse (*Apodemus agrarius*) also showed relatively high prevalence rates up to 10%, 25% and 20.8%, respectively in China.[199,231,232] In Korea, prevalence rates in black striped field mouse were up to 23.6%, hence this rodent species was suggested to be among the most important reservoir hosts in Asia.[233]

Anaplasma phagocytophilum has been detected in several other wild vertebrates including boars, foxes, bears, European bisons, donkeys, mooses, hares, Eurasian lynx, Coyotes, mountain lions, birds and reptiles. However, their role in the epidemiological cycle of the bacterium has not been assessed.[54,234,235] In the western USA, lizards and snakes were both seropositive and PCR-positive to *A. phagocytophilum,* but *I. pacificus* larvae fed on lizards did not acquire or transmit the bacterium, suggesting that reptiles can be naturally infected but unlikely to be competent reservoir hosts.[236] Raccoons (*Procyon lotor*), have been reported to be competent reservoir hosts for *A. phagocytophilum.*[224,237] In northwestern California, gray foxes (*Urocyon cinereoargenteus*) hosted all three life stages of *Ixodes* spp. ticks, displayed a high seroprevalence of 51% and PCR-positivity of 9% and urban foxes had the same seroreactivity rate than dogs. Therefore, gray foxes were considered as good sentinels for the bacterium transmission in this part of the USA.[238] Similarly, 25% of wild foxes (*Vulpes vulpes*) were PCR-positive for *A. phagocytophilum* in Austria.[239] Wild boars (*Sus scrofa*) are strongly suspected to be reservoir hosts for *A. phagocytophilum* human strains in Europe as some studies demonstrated that *A. phagocytophilum* isolates from these animals and humans harbored the same *groEL, ankA* and *msp4* gene sequences.[54,150,216] Furthermore, all three-life stages of *I. ricinus* can feed on wild boars.[216] However, other studies suggested that wild boars are capable to control *A. phagocytophilum* infection through activation of innate immune responses, phagocytosis and autophagy explaining the low prevalence in some European regions and making them less likely to be a competent reservoir hosts.[150,240,241] In some geographic areas, several bird species are thought either to be competent reservoir hosts or to contribute to the circulation and spread of infected ticks.[45,242-247]

3.5 Life cycle of Anaplasma phagocytophilum transmission by Ixodes tick species

All *Ixodes*-transmitted pathogens of humans need a vertebrate reservoir host for their perpetuation in nature.[54] More specifically, *A. phagocytophilum* is considered to be naturally maintained in complex and not fully assessed enzootic tick-wild animals cycles (Figure 9).[56] In the case of bacterial tick-borne infections that often lead to immune system stimulation in the reservoir host or to its death limiting the bacteriaemic phase, ticks represent a critical feature for the maintenance of the enzootic cycle in nature. The perpetuation of cycles can be ensured either by the transmission of the pathogens between different tick developmental stages (transstadial transmission), or between generation (transovarian transmission) or between ticks during cofeeding.[80,248,249]

The life cycle of *Ixodes* ticks lasts for almost two years[80] and its duration depends on climatic conditions varying from less than a year in tropical regions to three years or more in temperate regions.[250] This life cycle comprises four distinct developmental stages, i.e., egg, larva, nymph and adult. *Ixodes* ticks activity varies according to the life stage and they mostly quest on vegetation in prime suburban real estate.[80] Some authors consider only three life stages including larvae, nymphs and adults.[21,56] The feeding behavior at each life stage has a directly effect on the risk of tick-borne pathogens transmission.[80] All *Ixodes* species of public health relevance need to feed on a new host at each life stage after hatching except for males that do not feed, and the blood meal is completed in three to five days.[56,80] Ticks belonging to *I. persulcatus* complex are nonspecific feeding ticks that can have their blood meal either on various host reservoirs or on humans.[80]

Anaplasma phagocytophilum is transmitted to the host during the bite of a nymphal or adult tick infected during previous stages (larval or nymphal) (Figure 9).[35] Transmission of *A. phagocytophilum* to the host during tick feeding occurs usually within 24 to 48h.[251,252] As nymphs have very small size (approximately 1mm), they are often able to feed much longer on humans and are at increased risk to transmit tick-borne pathogens such as *A. phagocytophilum*.[80] In a recent study, 41% of retrieved ticks from humans in Italy were from nymphal stage.[253] Moreover, ticks have the capacity to modulate host immune and inflammatory responses that may also decrease the chance of detection.[254] Considering that *A. phagocytophilum* is transmitted transtadially in ticks, nymphs and adults contaminated in a previous stage last infected after molting and are able to contaminate susceptible hosts during the following blood meals.[80] Adult female ticks require an addition blood meal and are thus twice likely to acquire the infection.[21,54,80] As no transovarial transmission of *A. phagocytophilum* among *Ixodes* ticks occurs,[118] larvae are mostly considered free from infection until hatching and having their first blood meal.[54,80] Another consequence of the absence of transovarial transmission is the interruption of

A. phagocytophilum cycle when adult female tick lay their eggs.[255] However, transovarial transmission has been documented in moose ticks *Dermacentor albipictus* and seems to be due to an atypical feeding system as compared to normal *Ixodes* infection cycle.[179]

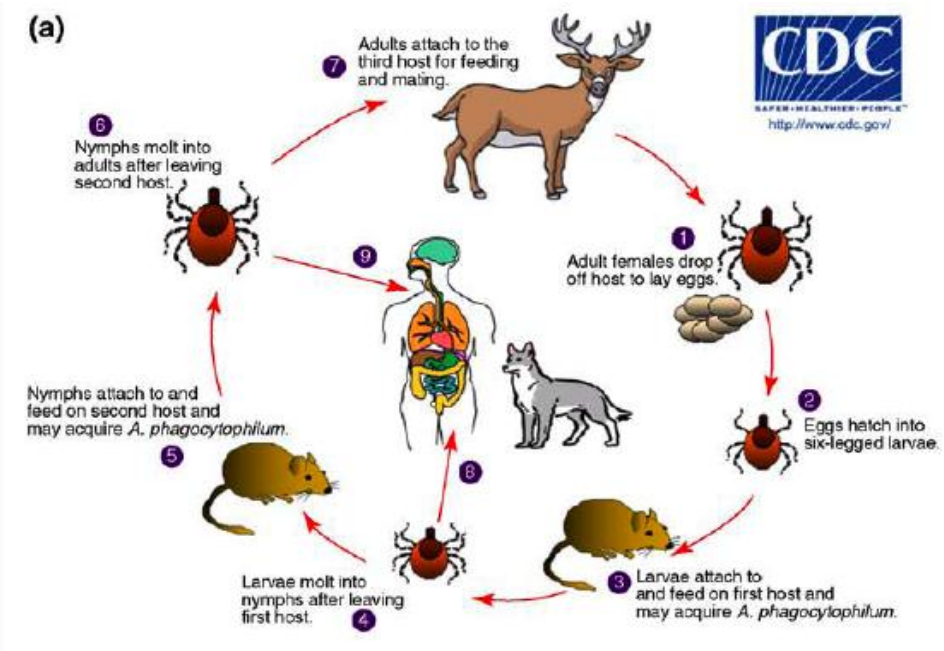

Figure 9. Transmission cycles of *Anaplasma phagocytophilum* in *Ixodes* spp. tick populations and infection of people and dogs. The pathogen is acquired from reservoir hosts during feeding by larval or nymphal ticks and then transmitted in subsequent feedings of nymphal or adult ticks.[255]

Ixodes tick species involved in the transmission of *A. phagocytophilum* in the USA, Europe and Asia are exophilic, telotropic and anthropophilic ticks. They have an open questing behavior, a wide host range and a ubiquitous distribution. Some of them such as *I. scapularis* are reported to have a high affinity for biting humans, hence are also able to transmit the bacterium from host reservoirs to people.[1,54] Therefore, the trophic preferences of these ticks are difficult to determine although it has been suggested that larvae parasitize small mammals while nymphs and adult stages are more likely to feed on medium (such as rabbits) and large mammals (such as ruminants), respectively.[56] Other more nidicolous and host-specific endophilic ticks are thought to play a role in niche cycles which may contribute to the persistence of the bacterium in nature.[54] Some recent studies showed that rodents could be reservoir hosts for *A. phagocytohilum* in an independent epidemiological cycle, involving only rodents as mammalian hosts. In the USA, two potential alternative *A. phagocytophilum* epidemiological cycles have been described, one involving *N. mexicana*, *P. maniculatus* and *I. spinipalpis* ticks.[176,177] and another involving cotton tail rabbit (*Sylvilagus spp.*) with *I. dentatus* and *I. scapularis*.[178] Similarly, red wood chipmunk hosts both antropophilic (*I. pacificus*) and nidicolous (*I. angustus*) ticks and is

suggested to maintain niche cycles.[257] In the UK and Central Europe, at least three independent epidemiological cycles have been described involving rodents with *I. trianguliceps*,[141,185,213,258,259] and hedgehogs with *I. hexagonus.*[215] These mammilian hosts can harbor two to three different stages of both endophilic and exophilic ticks simultaneously and thus promote the transmission to human through the anthropophilic ticks.[260]

3.6 Other transmission ways

Although *A. phagocytophilum* is primarily a tick-borne pathogen, other ways of transmission have been described including percutaneous and blood sub inoculation, blood transfusion, vertical and nosocomial transmissions.[164,261-263] Currently, eight human cases of transfusion-acquired granulocytic anaplasmosis have been reported, seven in the USA[164,264-268] and one in Slovenia.[269] Another probable transfusion-transmitted *A. phagocytophilum* infection has been described from the USA.[270] The seroprevalence of *A. phagocytophilum* among human blood donors in the USA ranges from 0.5% to 11.3% (Table 2).[271,272] In Europe, a very high prevalence rate has been reported in Greece with almost 21% of blood donors being seropositive for *A. phagocytophilum* (Table 2).[273] Because the risk of developing complications seems to be increased in some transfused people such as immunocompromised patients and because *A. phagocytophilum* can persist up to 18 days in refrigerated (4°C) human blood products, this infection is among the TBDs considered to represent a potential risk for transmission by blood transfusion in the USA.[274,265] Therefore, *A. phagocytophilum* should be suspected and researched in every transfused person who develops acute thrombocytopenia especially if associated with febrile illness and leucopenia. In addition, because sharing blood products between different areas is growing, such acute illness after blood transfusion might be included in the differential diagnosis even in nonendemic areas.[164,265,266,269,275]

Table 2. Seroprevalence of *Anaplasma phagocytophilum* in blood donors form the USA and several European countries.

Country	Number of blood donors	Prevalence (%)	Method	References
AMERICA				
USA				
Connecticut	992	3.5	IFA	271
Wisconsin		0.5		
Westchester County (NY)	159	11.3	IFA	272
EUROPE				
Poland	50	2.0	IFA	276
Eastern	32	9.4		277
Lublin	56	5.4		278
Bulgaria	70	2.9	IFA	279
Norway	301	16.2	IFA	280
Germany	103	1.9	IFA	281
Austria	357	9.0	IFA	70
Switzerland	530	1.1	IFA	282
Belgium	402	15.9	IFA	283
France	50	0.0	IFA	284
Greece	496	21.4	IFA	273
Portugal	96	4.2	IFA WB	285

IFA: immunofluorescence assay; WB: western blot.

In canine species, no cases of transfusion-transmitted granulocytic anaplasmosis have been recorded. A recent study carried in the UK screened 262 healthy canine blood donors without travel history outside of the country for several vector-borne pathogens by PCR, and none was positive for *Anaplasma* spp. Even though the UK is not an endemic region for *A. phagocytophilum* and its vectors, this bacterium is considered among the organisms of potential significance in transfusion medicine in this country.[286] Another study from the USA failed to detect positive individuals to *A. phagocytophilum* among 118 feline blood donors.[287] However, it has been strongly recommended to screen canine blood donors for *A. phagocytophilum* infection in endemic areas because some PCR-positive dogs can be clinically healthy and also because of possible chronic carrier status.[62,287,288] Finally the consensus statement on canine and feline blood donor screening for infectious disease of the American College of Veterinary Internal Medicine (ACVIM) recommends to test for diseases that meet at least three of the following criteria: (1) the infectious agent is known to induce clinical infections in recipients via blood transmission, (2) the infectious agent can cause subclinical infections making asymptomatic carriers possible accidental blood donors, (3) the infectious agent can be cultured from the blood of an infected

animal and (4) the disease induced in the recipient is severe or difficult to clear. The consensus statement also recommends considering testing in the case of documented experimental transmission without described clinical transmission via transfusion or if the disease does not represent a threat to the recipient or is easily cleared.[289]

Perinatal and transplacental transmissions have also been reported in people and cattle, respectively.[262,290] In dogs, no report described such transmission and a study on naturally infected bitch did not shown any perinatal transmission.[291] A study described the first nosocomial infection in people in China after direct contact with blood and respiratory secretions.[263] However, a recent report contradicts the nosocomial transmission of *A. phagocytophilum* in those patients based on discrepancies in clinical and laboratory features when compared to HGA cases from the USA and suggests that those Chinese patients could have been infected with a newly discovered bunyavirus, called 'severe fever with thrombocytopenia syndrome virus' (SFTSV).[292] Human cases of granulocytic anaplasmosis have been also described after percutaneous exposure or inhalation of contaminated blood of deer in the USA.[261] According to these previous reports, respiratory secretions could also be a source of infection. In a case of canine granulocytic anaplasmosis with respiratory signs, inclusions of *A. phagocytophilum* have been identified in neutrophils from tracheal wash smear.[291] Consequently, precautions might be taken when necropsies are performed on animals suspected of granulocytic anaplasmosis.[81]

3.7 Anaplasma phagocytophilum infection in humans

Several wild and domestic animals are receptive to *A. phagocytophilum.* However, the disease has been reported only in a few species including domestic ruminants, horses, cats, dogs and humans.[62,95,172,293-297] The first human granulocytic anaplasmosis (HGA) case has been reported in the USA in the mid-1990s.[143] In the USA, HGA is a nationally notifiable disease since 2000[298] and the number of cases has critically and rapidly increased between 2000 and 2012 from 348 to 2389 cases.[18,49] Data from the USA CDC and MMWR reported 10,670 human cases between 2010 and 2013, and a 8-fold increased number of reported cases between 2000 and 2013.[50] The disease incidence has increased from 1.4 to 6.3 cases per million persons per year between 2000 and 2010[293,298,299] and a 12-fold increased incidence was recorded between 2001 and 2011.[300] Figure 10 shows the evolution of annual cases of human granulocytic anaplasmosis in the USA from 1994 to 2010.

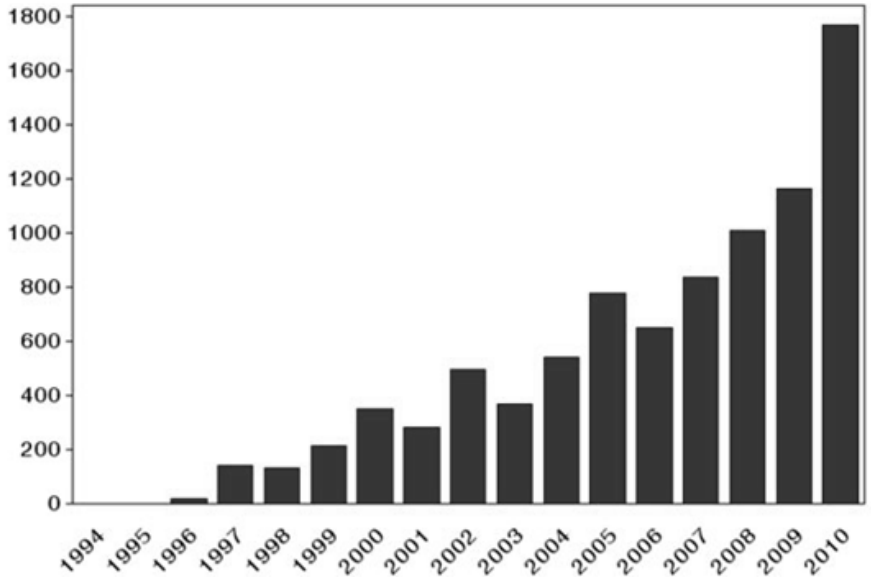

Figure 10. Number of annual human granulocytic anaplasmosis cases in the USA from 1994 to 2010 (http://www.cdc.gov/anaplasmosis/stats/).

HGA is currently considered the third most important VBD in both the USA and Europe and is also increasingly diagnosed in Asia.[76,204,301] Endemic regions include the Upper Midwest, New England, parts of the midAtlantic states and northern California in the USA and also several parts of Europe (central, northern and western countries) and some Asian countries.[73,78,92,302] Serological surveys carried in endemic areas of the USA found prevalence rates ranging from 15 to 36%.[271,303] Serological evidence of human exposure to *A. phagocytophilum* has been reported in almost all European countries with prevalence rates ranging from less than 1% to 32% and the disease has already been reported in several of them.[21,71,73,304,305] Similarly, exposure to *A. phagocytophilum* in China has continuously increased in high-risk populations according to the Tianjin CDC from 8.8% to 59.2% between 2006 and 2009.[77,306] Despite a moderate to high seroprevalence in several countries, HGA is still unrecognized and rarely diagnosed due to several factors including limited epidemiological, ecological, clinical and microbiological information, difficulties in the diagnosis, possible asymptomatic or subclinical infections and the lack of awareness by physicians and the public.[21,78] Seroprevalence studies conducted worldwide are summarized in Table 3.

Table 3. Seroprevalence of *Anaplasma phagocytophilum* in healthy, high-risk or sick populations worldwide.

Country	Number	Study population	Prevalence (%)	Method	References
AMERICA					
USA					
	9,987	Healthy military personnel	2.6	ELISA	299
			0.11	WB	
Wisconsin	475	Healthy permanent residents	14.9	IFA	303
New Jersey	202	People evaluated for Lyme disease	16.3	ELISA	307
Great Smoky Mountains and ROMO National Parks	141	Healthy permanent employees	8.1	ELISA	308
Peru					
Lima	160	Healthy urban residents	0.0	IFA	309
Northern coast, southern Peruvian Andes and Peruvian jungle region		Healthy rural residents			
EUROPE					
Poland					
	180	Patients suspected for rickettsiosis	4.9	IFA	310
Eastern and southern	216		29.6	IFA	302
Eastern	400	Employees of National Forest	8.0	ELISA	311
		Healthy client-owned dogs	2.8	PCR	
Northern and northeastern	478		9.6	IFA	312
Eastern	39	Raworkers from forest areas	5.1	IFA	277
	119	Farmers	11.8		
Southeastern	113	Forestry workers	17.7	IFA	278
Northeastern	130	Forestry workers	6.2	IFA	276
		People living in Białowieża Primeval Forest			
Bulgaria	200	Patients with a history of tick bites	7.4	IFA	279
Slovenia	53	Children with fever and tick bite	1.9	IFA	313
Koster Islands	185		11.4	IFA	314
	90	Permanent residents	17.0	IFA	315
		People bitten by ticks			
Czech Republic	809	Patients suspected of tick-borne encephalitis	9.9	IFA	208

Norway	47	Patients with clinical signs and history of tick bite	29.8	IFA	316
UK	518	Farmworkers and family members	1.5	IFA	254
The Netherlands	108	Febrile patients with unresolved etiology	4.0	IFA	304
	174	Patients suspected of Lyme disease	4.0		
	154	Forestry workers	1.0		
	54	Healthy controls	0.0		
	626	Patient with a tick bite or erythema migrans	0.8	PCR	317
Belgium	1,350	Patient with clinical signs compatible with a TBD	31.0	PCR IFA	73
	148	Workers professionally exposed	8.1	IFA	283
Germany	150	Forestry workers	14.0	IFA	281
	105	Patients with Lyme disease	11.4		
Bavaria region	107	Patients with history of tick bite	7.5	IFA	318
Switzerland	181	patients with suspected tick-borne encephalitis and healthy	17.7	IFA	319
Northern	70	controls	17.1	IFA	284
Eastern	258		9.0	IFA	320
	149	People bitten by *Ixodes* ticks	12.7		
		Hunters			
	205	Persons previously diagnosed with Lyme disease	19.5		
		Patients previously diagnosed with tick-borne encephalitis virus			
Cyprus	227	Farmers, or workers in farms, people in contact with animals of veterinary importance and/or ticks	32.0	IFA	71
Italy	181	Forestry rangers	8.8	IFA	305
			0.6	WB	
Portugal	147	Patients with Lyme disease, forestry workers, and persons with history of tick bite	1.4	IFA	321

	367	Potentially exposed patients	5.8	IFA	285
	792	Clinically ill patients	3.9	IFA	322
Turkey	637		7.8	IFA	323
ASIA					
China					
Eight provinces	3,669	Healthy people living in forest	7.11	IFA	324
Nine provinces	7,322	areas	15.4	IFA	78
Two provinces	819	Healthy agrarian individuals	1.5		
Beijing	562	Healthy urban residents	14.1	IFA	201
Yiyuan County	46	Healthy farmers from rural	26.7	IFA	204
Near Tianjin	365	areas	8.8	IFA	77
Central and Southeastern	323	Healthy farmers	20.0	IFA	325
		Healthy Farmers			
		People at high risk exposure to ticks and animals			

IFA: immunofluorescence assay; ELISA: enzyme-linked immunosorbent assay; PCR: polymerase chain reaction; WB: western blot.

High-risk populations (i.e., people living in forest areas and forestry workers, people living in rural areas and farmers, hunters, national parks rangers, military personnel, people in close contact with domestic animals, and people at high risk of exposure or previously exposed to ticks) have significantly higher prevalence rates of *A. phagocytophilum* exposure.[78,278,299] The disease is typically seasonal with most cases recorded during spring and summer. Major risk factors for acquing *A. phagocytophilum* infection include outdoor activities especially related to wooded areas, meadow habitats and grasslands, immunodepression and blood transfusion.[35,253] HGA is an unspecific flu-like illness mostly characterized by fever, headache, chills, myalgia and malaise.[79,92,255] Symptoms usually appear five days to three weeks after a tick bite.[265] Less frequently, human patients can display arthralgia, rash, liver injury, digestive (nausea, vomiting, diarrhea), respiratory (cough, pulmonary infiltrates, acute respiratory distress syndrome) or nervous signs (stiff neck, confusion).[79,92] Clinical signs are frequently accompanied by nonspecific hematological and serum biochemistry profile modifications including thrombocytopenia, leukopenia, lymphopenia, anemia and increased liver enzymes activity.[79,92,255] Leukopenia and lymphopenia can lead to severe opportunistic infections such as herpes simplex esophagitis, *Candida albicans* pneumonitis/esophagitis and invasive pulmonary aspergillosis.[326-329] The severity of the disease and mortality are strongly correlated with advanced age of patients, immunosuppression, the presence of co-morbidities and delayed onset of treatment.[316,330] The differential diagnosis should include other acute viral and bacterial infections, some inflammatory disorders, other vector-borne diseases and malignancies (Table 4).[300]

Table 4. Differential diagnosis of human granulocytic anaplasmosis.[255,300]

Viral infections	Enterovirus infection, Epstein-Barr virus, Hantann virus, human herpes virus-6, human parvovirus B19 infections, viral hepatitis A, B, C
Bacterial infections	Acute bacterial endocarditis, group A streptococcal infection, leptospirosis, meningococcemia, *Mycoplasma pneuminiae*, Neisseria gonorrhea sepsis, Neisseria menongitidis sepsis, post-group A streptococcal infection, Q fever, rat-bite fever, secondary syphilis, septic shock syndromes, typhoid fevers
Other VBDs	African tick-bite fever, babesiosis, bartonellosis, chikungunya virus disease, Colorado tick fever, *Ehrlichia muris*-like agent infection, human granulocytic ehrlichiosis *(E. ewingii)*, human monocytic ehrlichiosis *(E. chaffeensis)*, heartland virus fever, Lyme disease, malaria, murine typhus, Rocky Mountain spotted fever, severe fever with thrombocytopenia virus infection, scrub typhus, tularemia, dengue virus fever, malaria, Powassan virus disease/tick-borne encephalitis, West Nile fever
Inflammatory disorders	Allergic-drug reactions, idiopathic thrombocytopenia purpura, immune complex-mediated illnesses, Kawazaki syndrome, thrombotic thrombocytopenic purpura, toxic hemophagocytosis, macrophage activation syndromes
Malignancies	Lymphoma, acute leukemia

In most cases of HGA, clinical signs are mild and self-limited, with favorable evolution even without treatment. People usually recover completely after antibiotic therapy however some patients could display persistent clinical signs from one to three years after treatment.[79,92,331] Life-threatening complications have been reported to occur in 3% of patients (Table 5).[293] Two reports from China, described complications by systemic inflammatory response syndrome (SIRS) and multiple organs deficiency syndrome (MODS) in 45.8% and up to 41.2% of cases.[78,301] Consequently, half of the HGA cases are hospitalized and up to 17% require intensive care unit admission especially when diagnosis and treatment were delayed.[79,293,327,332-334] Due to the potential serious outcome associated with the disease, the Infectious diseases Society of America recommends to give antimicrobial therapy to every person suspected to have HGA on the basis of the clinical presentation although mild or self-limiting pending the laboratory results and to not delay treatment.[255,292] Even though relatively high hospitalization rates are recorded in some studies, the fatality rate is usually lower than 1%.[300] However, mortality rates up to 8.1 and 10% were recorded in China and the USA, respectively.[21,78] Two reports from China, described 3.2% and 26.5% of fatality.[263,301]

Table 5. Complications and associated risk factors in human granulocytic anaplasmosis.[78,92,300,301]

Clinical complications
Hemodynamic Toxic or septic shock-like syndrome, coagulopathy, hemorrhage, myocarditis, pancarditis, renal failure, systemic inflammatory response syndrome (SIRS), multiple organ deficiency syndrome (MODS)
Respiratory Pneumonia, acute respiratory distress syndrome (ARDS)
Nervous system Meningoencephalitis, cranial nerve palsies, demyelinating polyneuropathy, brachial plexopathy, seizure
Others Rhabdomyolysis, opportunistic infections, acute abdominal syndrome
Risk factors
Preexisting disease Immunosuppressive conditions

3.8 Epidemiological role of dogs

Although dogs are susceptible to *A. phagocytophilum*, they are mostly recognized as incidental hosts and their role as potential reservoirs is still controversial.[136,335] Dogs are considered unlikely reservoir hosts due to the potential short duration of bacteremia (< 28 days) and uncertainty regarding their ability to host enough nymphal tick stages to contribute to the spread of the bacterium.[10,54] In Austria, no significant difference in the seroprevalence of *A. phagocytophilum* among owners of seropositive pets and owners without pets was observed, suggesting that pets are not a source of infection for humans.[336] However, according to some authors, almost all studies investigating the role of dogs in the transmission of TBDs focused on companion dogs. These animals are usually treated against ectoparasites, have limited free access to the outdoors and reservoir host's habitats, and are less exposed to ticks when compared to hunting, stray or shelter dogs. Therefore, these studies may not accurately reflect the public health risk associated with dogs in endemic areas.[337] Others suggested that domestic animals including dogs could be considered as potential reservoir hosts of *A. phagocytophilum* in Europe especially in urban areas.[337-340] In a study from Hungary, the prevalence of *A. phagocytophilum* DNA in stray dogs was higher than in several studies from other European countries.[337] In addition, two studies reported high prevalence rates of *A. phagocytophilum* DNA in dogs suspected to have Lyme disease and rural dogs from Poland and China, respectively.[201,341] *Anaplasma phagocytophilum* was also the most frequently detected bacterium by PCR in stray dogs that lived in close contact with domestic animals and humans in rural and peri-urban areas of the Mediterranean zone of Jordan.[342] In addition, high prevalence rates of *A. phagocytophilum* DNA was found in *I. ricinus* collected from dogs in

Belgium and Poland, and *Rhipicephalus sanguineus* (adult and nymphs) from free-roaming dogs in Egypt.[84,343,344] Moreover, *A. phagocytophilum* DNA was detected in experimentally infected dogs during 60 days without immunosuppressive drug, and the canine immune response seems to have evolved to only partially control infection, suggesting a longer bacteremia that possibly allow timely transmission to the vector.[117,161] Based on these results, dogs could act as potential reservoir hosts for the bacterium in some regions, but further studies are needed to confirm this hypothesis.

The geographical distribution of canine infection seems to parallel the distribution of HGA in the USA with a positive association of human and canine cases in many states.[46,345] Indeed, several studies found the highest prevalence rates of *A. phagocytophilum* antibodies in dogs from the upper Midwest, Northeast and Mid-Atlantic, which correlate with areas where the highest incidence rates of human anaplasmosis were reported.[46,50,293,345-347] In addition, the estimated regression coefficient for the endemic risk factor in the contiguous USA model was positive and significant. This implies higher prevalence among dogs living in areas where HGA is endemic.[347] Furthermore, human and canine strains of *A. phagocytophilum* were similar according to several gene sequencing, and human isolates have been reported to induce clinical disease in dogs in both Europe and the USA.[120,150,159-163] Therefore, in addition to the possible role of dogs as potential reservoir hosts, the prevalence data of *A. phagocytophilum* infection in dogs provides important information on the incidence, risk factors, sources of exposure, and real-time risk of exposure for human infection.[46]

4. *Anaplasma platys*

4.1 Transmission

The natural mode of transmission of *A. platys* has not been demonstrated conclusively, but it likely involves a tick vector. This bacterium is most likely transmitted through *R. sanguineus* tick bites although the tick vector competency has not been proven.[89,90] Indeed, although one experimental study failed to demonstrate the ability of *R. sanguineus* to transmit *A. platys*,[348] its DNA has been frequently detected in this tick species. In addition, the *16S rRNA* gene fragments amplified from ticks were identical to *A. platys* sequences obtained from dogs infested by these ticks and canine infection with this bacterium is common in areas with high *R. sanguineus* pressure.[349-361] Moreover, *A. platys* has been repeatedly reported from areas where other *R. sanguineus*-transmitted pathogens such as *Ehrlichia canis*, *Babesia canis* or *Rickettsia conorii* are commonly present[362-364] and coinfection between *A. platys* and these agents have been reported.[365-371] A recent study detected *A. platys* DNA in adult and nymph *R. sanguineus* ticks collected from negative dogs and did not found any difference between *A. platys* detection in ticks collected from positive and negatives dogs. These findings suggest that these tick stages may acquire the bacterium in the previous life stage and may maintain a constant load after moulting. Therefore, as *R. sanguineus* ticks display a three-host life cycle (i.e., each life stage requires a new host to feed on), a transstadial transmission of *A. platys* may occur, possibly playing an important role in the pathogen spreading throughout canine populations.[358]

Anaplasma platys DNA has been detected in several other *Rhipicephalus* spp. ticks such as *R. camicasi*, *R. turanicus*, *R. evertsi* and *R. bursa*.[360,372-375] In addition, the DNA of this bacterium has been detected in several other tick species including *Haemaphysalis longicornis*, *H. leachi*, *I. persulcatus*, *Hyalomma* spp., *Ampblyomma* spp.[233,360] and in the dog chewing louse *Heterodoxus spiniger*.[376] However, further studies are needed in order to confirm their role as competent vectors of *A. platys*.[360] *Dermacentor auratus* could be a competent vector of *A. platys* in some Asian countries.[90,352]

Similarly to other *Anaplasma* species, *A. platys* can be transmitted through direct blood subinoculation.[117,364,377-379] Therefore, because transmission of these pathogens via infected blood can occur and asymptomatic infections are frequent, screening canine blood products for bacterial DNA with a PCR assay is recommended in highly endemic areas to ensure the safety of blood products.[99,380] A recent study detected *A. platys* DNA in uterine, ovarian and fetal tissue samples from both pregnant and non-pregnant naturally infected bitches, suggesting possible vertical transmission of this infection in canine species.[381]

4.2 Reservoir hosts and epidemiological role of dogs

Dogs are considered the main reservoir host of *A. platys* and are also a strongly preferred host for *R. sanguineus*.[112,378,382] *Anaplasma platys* has been detected in all stages of *Haemaphysalis longicornis* and *I. persulcatus* ticks collected from small wild-caught mammals and striped field mouse (*Apodemus agrarius*) was found infected by this bacterium with a prevalence of 16% in Korea.[233] Similarly, 14.5% of wild foxes were infected by *A. platys* in Portugal.[383] These two studies suggest that some wild animals may play a role in the epidemiology of this infection and could act as candidate reservoir hosts.

4.3 Zoonotic potential of Anaplasma platys

For decades, *A. platys* was thought to infect dogs exclusively.[40] However, recent reports described this infection in domestic ruminants,[384,385] cats[386,387] and even in humans.[39-41] Camelids (*Camelus dromedarius*) infection by *Anaplasma* species closely related to *A. platys* in both Tunisia and Saudi Arabia were also recently reported.[388,389] Another study detected *A. platys* DNA in Camelids in Nigeria.[390] Previous reports have described intraplatelets inclusions resembling those of *A. platys* in a stained blood film from a cat in Brazil[391] and organisms within platelets of an impala in South Africa identified by transmission electron microscopy on blood.[392] In addition, organisms with 99.5% and 100% gene sequences homology with *A. platys* were identified from blood samples from sheep in South Africa[393] and goats in Cyprus,[394] respectively.

Infection with *A. platys* was suspected in people from Venezuela based on the appearance of inclusions in platelets in stained blood films. Indeed, between 1993 and 2012, 5,954 people had intra-platelet inclusions in buffy coat smear and most of these patients displayed moderate to severe clinical signs, some were hospitalized, and some patients responded well to tetracyclines, especially to doxycycline. When platelet-rich plasma from buffy coat smear-positive cases was prepared for

ultrastructural examination by transmission electron microscopy, and organisms compared with ultrastructural studies described in the United States[111] and in Venezuela, it was concluded that organisms infecting dogs and people appeared different. In canine organisms, a well-defined double membrane, characteristic of the Anaplasmataceae family, was evident and the intra-vacuolar space was clear, whereas in organisms from human cases, organism membranes were thickened and the intra-vacuolar space appeared electron-dense. To date, the etiology of these intra-platelet organisms has not been identified.[40,395] Similarly, intra-platelets morulae were identified on blood smears from HIV-seropositive patients in Venezuela and showed morphological characteristics similar to those observed in infected dogs confirmed by PCR. However, the *Ehrlichia* or *Anaplasma* species involved was not identified.[396]

4.4 Genetic diversity

Comparison between experimental and natural *A. platys* infections in dogs revealed morphological and ultrastructural variations that have been associated with different developmental stages of *A. platys* but may also suggest differences between strains.[106,108] Molecular analysis and variations in clinical severity also supports the possibility of multiple *A. platys* strains associated with geographic variation.[108,397-399] Indeed, Infectious canine cyclic thrombocytopenia caused by *A. platys* infection is usually mild and self-limited especially in the USA and Australia.[400,401] In the USA, although some clinical signs have been described, most reports of experimental and natural infections have indicated that *A. platys* causes no or few clinical signs in dogs.[99,108,400,402,403] In contrast, experimental infection using an *A. platys* Greek strain seems to be more virulent than the inoculation with American strains.[404] Similarly, *A. platys* natural infections were more frequently associated with severe and life threatening clinical signs, absence of response to treatment and mortality in Mediterranean and South American countries including France, Greece, Spain, Italy, Croatia, Portugal, Israel, Chile, Turkey and Tunisia.[86,371,382,397,401,404-408] Although variations in pathogenicity could be caused by *A. platys* strains diversity, other factors can explain the variability in clinical signs including concurrent diseases and more specifically co-infections with other VBPs or intrinsic factor such as genetic factors, immune status of the animal and stress conditions.[365,366,368,379,397,401] Some authors suggested that the genetic diversity of *A. platys* might be lower than the reported diversity of *A. phagocytophilum* possibly due to restricted movement of infected hosts and/or the limited host range of *A. platys*.[398] Therefore, although a variety of polymorphisms has been reported among *A. platys* strains of different geographic origin, there is little genetic diversity among this species[100,409] and this variability may also be associated with the range of hosts within a specific country.[399]

5. Distribution and prevalence of *Anaplasma phagocytophilum* and *Anaplasma platys* in dogs

Both *A. phagocytophilum* and *A. platys* have worldwide distributions. Endemic areas of *A. phagocytophilum* include some regions of the USA (northeastern and mid-Atlantic, Upper Midwest, and Pacific Northwest states), Europe and Asia (China, Siberian Russia, and Korea). These regions correspond to occurrence areas of *I. persulcatus* group ticks.[9,92,136,410,411] *Anaplasma platys* has been reported in all continents but is mainly present in tropical and subtropical regions such as southern USA, South America, the Mediterranean area including southern Europe and North Africa.[86,106,112,354,369,382,404,406,412-418] It is also prevalent in other African and Asian countries[233,350,355,366,367,405,409,419-422] and has been reported in Australia.[370,423] Several prevalence studies on both bacteria have been conducted in dogs in various American, European, Asian and African countries and are summarized in Tables 7 to 10. However, data are lacking in large parts of Asia, Africa, South America and Australia especially for *A. phagocytophilum*. The geographic variation in tick exposure, the differences in inclusion criteria to select canine populations, and the use of different serologic test (i.e., immunofluorescent antibody test, enzyme-linked immunosorbent assay or Western blot) make comparison between studies difficult.[81,288,357]

The first canine granulocytic anaplasmosis (CGA) cases in the USA were detected in California; therefore, the exposure of dogs to this organism has been recorded in more than 39 USA states and highest rates were noted in the upper Midwestern, northeastern and western states. Serological surveys revealed prevalence rates ranging from 0% to 40%.[46,49,307,345-347,411,424-432] Infectious canine cyclic thrombocytopenia (ICCT) caused by *A. platys* has been first documented in the USA in 1978.[99] Five countrywide serologic studies showed an overall prevalence of *Anaplasma* spp. of 1.9% to 4.8% with the highest rates recorded in northeastern regions.[46,432,345-347] One of these studies used species-specific peptides to detect canine antibodies to *A. phagocytophilum* and *A. platys* with prevalence rates of 3.5% and 1.5% in the USA, 1.1% and 1.8% in Canada and 3.4% and 10.3% in the Caribbean, respectively.[46] In addition, cases confirmed of CGA[9,136,410,411,433-436] and of ICCT[99,400,402] were confirmed in several USA states. In Canada, three serologic surveys on *Anaplasma* spp. are available (Table 7),[43,46,437] and four cases of CGA from Vancouver Island[438] and Saskatoon[439] were confirmed by DNA detection. In addition, a case report described a coinfection with *B. canis*, *E. canis* and *A. platys* in a dog imported from the Bahamas to Canada.[440] In Latin America and the Caribbean, the seroprevalence of *Anaplasma* spp. ranges from 1.0% to 53.2%.[441,442] In these regions, *A. platys* seems to be the most prevalent *Anaplasma* species with DNA detection rates among canine populations up to 48.8% in Brazil.[443] However, some studies and a case report have also detected the DNA of *A. phagocytophilum*

(Table 7).[180,340,444] Recently, a report from Colombia detected *A. platys* and *Anaplasma* spp. closely related to *A. phagocytophilum* in canine blood samples.[445]

Table 7. Prevalence of antibodies to *Anaplasma* spp., DNA detection of *Anaplasma phagocytophilum* and *Anaplasma platys* in blood samples from dogs in American countries.

American countries	**Number of dogs**	**Serology *Anaplasma* spp**		**PCR *A. phagocytophilum* (%)**	**PCR *A. platys* (%)**	**References**
		%	**Method**			
Canada	86,251	0.19	ELISA			43
7 provinces	285	1.1	ELISA			46
South Ontario, Quebec	53	0.0	ELISA			437
USA	3,950,852	3.8	ELISA			347
	3,588,477	4.4	ELISA			345
	479,640	4.8	ELISA			346
	14,496	1.9	ELISA			432
	6,268	1.5 - 3.5	ELISA			46
Oregon, California	2,431	2.4	ELISA			429
North Carolina, Virginia	1,845	1.1	IFA			426
Maine	1,087	7.1	ELISA			430
California	1,082	8.7	IFA			6
	182	40.0	IFA			9
Minnesota	731	55.4	IFA	7.2		411
	273					411
Oklahoma	259	33.0	IFA	9.5	0.0	424
Northern Arizona	233	11.6	ELISA			428
New Jersey	202	9.4	ELISA	0.0	6.9	307
North Carolina	118	0.0	ELISA			431
	27					365
Connecticut, New York	106	9.4	IFA, WB	11.1	33.3	425
Brazil	320				7.2	446
	60				1.6	447
	256				16.4	448
	230				15.6	449
	221				14.9	450
Rio de Janeiro	398					180
	253			6.0		340
Southeastern	198			7.1		451
Southern	196	9.7	ELISA	0.0	14.1	452
Central-northern Parana	138	13.8	ELISA			453

Northeastern	205				48.8	443
Puerto Rico	629	1.0	ELISA			441
Colombia	498	33.0	ELISA			454
Northern	218	53.2	ELISA		16.1	442
Uruguay	191				4.2	455
Nicaragua	39				13.0	456
Argentina	86				20.9	457
Bueno Aires	52				13.5	357
Mexico	1,706	9.9	ELISA			458
	100				31.0	459
Panama	201				21.4	460
Venezuela	43			0.0	16.3	461
Chile	30				20.0	382
French Guiana	65				15.4	417
Haiti	210	17.6	ELISA			462
	207			0.0	6.3	
West Indies	157	10.8	ICG		2.5	463
	110				4.0	464
Costa Rica	300				6.3	465
	146				10	466
Cuba	100				16.0	361
Caribbean region	29	10.0	ELISA			46

IFA: immunofluorescence assay; ELISA: enzyme-linked immunosorbent assay; PCR: polymerase chain reaction, WB: western blot; ICG: immunochromatography.

In Europe, seroprevalence to *Anaplasma* spp. has been reported in almost all countries with rates ranging from 1.1% to 56.5%.[322,338,467-470] The detection of *A. phagocytophilum* DNA has also been reported mostly from central and northern countries (Table 8) with prevalence rates up to 14.2%.[341] Additionally, several cases of CGA have been described.[60-66,471-474] In contrast, information is limited regarding the prevalence of *A. platys* infection in dogs from Europe, based on molecular analysis[288] but this infection seems to be emerging in this continent.[475] Most studies available are from southern countries with prevalence rates of *A. platys* DNA detection ranging from 0.4% to 57.7%.[476,477] In addition, several cases of ICCT have been reported from Croatia, Romania, Italy, Spain, Portugal and France[397,401,407,408,478] and a case of coinfection with *A. platys* and *B. canis* imported from Spain to Belgium.[479]

Table 8. Prevalence of antibodies to *Anaplasma* spp., DNA detection of *Anaplasma phagocytophilum* and *Anaplasma platys* in blood samples from dogs in European countries.

European Countries	**Number of dogs**	**Serology *Anaplasma* spp**		**PCR *A. phagocytophilum* (%)**	**PCR *A. platys* (%)**	**References**
		%	**Method**			
Germany	5,881	21.5	ELISA			480
	1,124	50.1	IFA			467
	522	43.0	IFA	5.7		481
	111	43.2	IFA	6.3		61
Northeast	1,862	17.8	IFA			482
	448	19.4	ELISA			483
Southern	171	50.3	IFA			470
	57	24.6	IFA			
Brandenburg	1,023			1.5		484
Russia						
European part	440	1.1	ELISA			469
Voronezh Reserve	82	34.1	ELISA			469
Hungary	1,305	7.9	ELISA			485
	199	10.6	IFA	1.9		486
Slovakia	87			8.0		487
	180	11.7	ELISA			488
Bulgaria						
Central-southern	167	19.2	IFA			489
Austria	1,470	56.5	IFA			490
United Kingdom	120			0.8		491
Sweden	611	17.7	IFA			492
	100	17.0	IFA			493
Finland	390	5.3	ELISA	0.5		494
Albania	30	40.0	IFA	0.0		495
Tirana	602	24.1	IFA	1.0	3.3	496
Latvia	470	0.85	ELISA			497
Romania	1,146	5.5	ELISA			498
	121	7.4	IFA			486
	109			2.7		486
	357			5.3		499
Southeastern	257	6.2	ELISA			500

Serbia	84	15.5	IFA			501
Poland	3,094	12.3	ELISA			502
Eastern	400	8.0	ELISA	2.8		311
Northwetern	192			1.0		503
	100			14.0		341
	92	14.0				
	50	0.0				
Central	79			1.3		504
Czech Republic	296	26.0	IFA	3.4		505
Italy						
Stretto di Messina	249	38.0	IFA			506
	5,881	32.8	IFA			507
	200			0.0	3.5	508
Central Italy	1,965	4.7	IFA			509
	1,232	8.8	IFA			510
	215	14.8	IFA	0.9	4.0	511
Sicily	344			0.0		398
	87	45.0	IFA		4.0	512
	344			0.0		
	87	44.8	IFA			338
	2			0.0		
Southern	165	37.6	IFA		2.3	477
	170				30.4-57.7	
	23-29				52.9	514
	34					358
Northeastern	338	4.7	IFA	0.0		515
	150	3.3	IFA	0.0		
Portugal	1,185	4.5	ELISA			516
	49			0.0	14.3	508
	55	55.0	IFA	0.0	9.1	322,468
Southern	100	16.0	IFA			517
	1,010				0.4	476
France	919	2.7	ELISA			518
Spain	466	11.5	IFA			8
Nothwestern	1,100	3.1	ELISA			519
	479	5.0	IFA			520
Grenada	73				19.2	521
Turkey	757				0.5	359
Thrace region	400			4.0	6.0	375

IFA: immunofluorescence assay; ELISA: enzyme-linked immunosorbent assay; PCR: polymerase chain reaction.

In Asia, *Anaplasma* spp. seroprevalence is available from China, Korea, Malaysia, Taiwan and Israel and range from 1.2% to 24.7% (Table 9).[522,523] *Anaplasma phagocytophilum* and *A. platys* DNA have also been detected in dogs with prevalence rates up to 39.5%and 32%, respectively (Table 9).[342,350] Case reports of ICCT have also been also described in Japan.[421,524]

Table 9. Prevalence of antibodies to *Anaplasma* spp., DNA detection of *Anaplasma phagocytophilum* and *Anaplasma platys* in blood samples from dogs in Asian countries.

Asian Countries	**Number of dogs**	**Serology *Anaplasma* spp**		**PCR *A. phagocytophilum* (%)**	**PCR *A. platys* (%)**	**References**
		%	**Method**			
Japan	154			0.0		420
	200				32.0	350
China	600	0.5	ELISA			
	243			0.4	0.0	525
	219	10.0	IFA	10.9		201
	162				0.0	526
	26	7.7	ELISA			527
Korea	1,058			0.1		528
	418	1.2	ELISA			523
	229	18.8	ELISA			529
	182	4.4	ELISA	0.0	0.0	522
		24.7	IFA			
	63			0.0		
Malaysia	48	9.3	ELISA	4.3		530
	30				13.3	371
Cambodia	101				0.0	531
Thailand	181				4.4	532
Philippines	70				0.0	533
Taiwan	344	5.2	ELISA			534
India	191	4.7	ELISA			535
	525				6.5	536
Israël	195	9.0	IFA			537
Jordan	38			39.5		342

IFA: immunofluorescence assay; ELISA: enzyme-linked immunosorbent assay; PCR: polymerase chain reaction.

In Africa, only a few prevalence studies have been published on *Anaplasma* spp. in dogs (Table 10). Seroprevalence rates recorded in African countries range from 11.8% to 47.7% (Table 10).[88,538] Considering that *A. platys* seems to be the most prevalent species in African countries[81] most molecular studies focused on this bacterium. Its prevalence among canine populations in Africa ranges from 1.2% to 20.4% (Table 10).[539,540] In contrast, very limited studies have investigated *A. phagocytophilum* infection in dogs in this continent. The DNA of this bacterium has been detected in Tunisia, Nigeria, Cape Verde and South Africa (Table 10).[86,541-543] In addition, an *Anaplasma* species closely related to *A. phagcytophilum* was detected in blood samples from South African dogs based on the *16S rRNA* gene sequencing[544] whereas all dogs from Algeria, Ghana and Maio Island tested by PCR were found negative (Table 10).[88,538,545]

In Australia, very few studies are available including one combining *Anaplasma* spp. seroprevalence and *A. platys* DNA detection[546] and three other *A. platys*-molecular based studies (Table 10).[370,546,547] Currently, no report on the occurrence of *A. phagocytophilum* is available from this continent.

Table 10. Prevalence of antibodies to *Anaplasma* spp., DNA detection of *Anaplasma phagocytophilum* and *Anaplasma platys* in blood samples from dogs in Africa and Australia.

Africa and Australia	**Number of dogs**	**Serology *Anaplasma* spp**		**PCR *A. phagocytophilum* (%)**	**PCR *A. platys* (%)**	**References**
		%	**Method**			
AFRICA						
Tunisia	286	25.2	IFA			86
	228			0.9	4.4	
Algeria						
Algiers	213	47.7	IFA	0.0	14.1	88
Tizi Ouzou, Bejaïa	110				5.5	418
Nigeria	245			0.8		542
	181				6.6	549
Senegal	34				2.9	550
South Africa	141			2.1		543
Ghana	17	11.8	ELISA	0.0	5.9	538
Côte d'Ivoire	140				8.5	360
	137				1.5	539
Cape Verde						
Priai	57			1.8		541
	130				7.7	551
Maio Island	153			0.0	3.3	545
Kenya	86				18.6	360
Gabon	255				1.2	539
Angola	103				20.4	540
AUSTRALIA						
	39				51.0	547
	215				10.0	370
	230				21.3	548
	238	3.5	ELISA		3.8	546

IFA: immunofluorescence assay; ELISA: enzyme-linked immunosorbent assay; PCR: polymerase chain reaction.

Cross-reactivity between *Anaplasma* spp. pathogens is reported to occur for both IFA and ELISA.[112,291,346,365,426,429,522] Therefore, in regions were both pathogens could be present (southern USA states, southern Europe, South America, Asia, and Africa), seropositivity may not necessary reflect exposure to *A. phagocytophilum* or *A. platys* and potential overestimation of their true prevalence and distribution can occur.[81,112,346,365,375,428,450,496] As a result, PCR-based assay is required to determine which of the two agents is responsible for positive serologic test results in regions where both bacteria are present.[112] In areas where the *Ixodes* tick vector is less prevalent or absent, a positive *Anaplasma* spp. serologic result could be the result of *A. platys* exposure.[498] Less frequent and minor serological cross-reactions were described at low titers between *A. phagocytophilm* and *Ehrlichia* species (i.e., *E. canis*, *E. chaffeensis*, *E. ewingii* and *E. sennetsu*), especially with hyper immune sera, when using IFA and immunoblot assay.[101,111,410,425,426,553,554] However, it is not clear whether the cross-reactivity with *E. canis* was attributable, in part, to antibodies against *A. platys* because dogs are sometimes exposed to both *E. canis* and *A. platys.*[498,552] In contrast, no cross-reactivity has been documented between *Anaplasma* spp. and *Ehrlichia* spp. when using the point-of-care dot ELISA.[81,552]

6. Conclusion

Vector-borne diseases are of growing concern worldwide because of their extending distribution and impact on human and animal health. These diseases are not prevalent in tropical regions only since some of them are widely distributed or mainly found in Europe and the USA.

Anaplama phagocytophilum and *Anaplasma platys* are two tick-borne bacteria currently known to infect both humans and dogs and displaying wide geographic distributions that overlap in some regions of the world. These two bacteria are responsible of canine granulocytic anaplasmosis and Infectious canine cyclic thrombocytopenia in dogs, respectively. Human granulocytic anaplasmosis, caused by *A. phagocytophilum*, is increasingly recognized worldwide with possible transmission via blood transfusion and frequent clinical complications requiring hospitalization. Although *A. platys* has been reported to infect people its ability in causing disease in humans has not been described.

Several epidemiological data are published worldwide on both bacteria. However, information is lacking on their respective prevalence in several countries, the competent vector of *A. platys*, the ability of tick species other than *Ixodes* spp. to transmit *A. phagocytophilum* and the reservoir host range of both bacteria especially in some regions such as Africa, Latin America, Australia and large parts of Asia. It is obvious that the transmission cycle of *A. phagocytophilum* is complex and not fully elucidated, and variations of the tick species and the reservoir host range exist according to the geographic location. Geographic variability in pathogenicity and severity of clinical signs also occur for both *A. phagocytophilum* and *A. platys* and could be explained by genetic variability. Due to these geographic variations, epidemiological data within a specific region are necessary to assess the risk of infection for dogs and humans and to sensitize local physicians on the presence of these pathogens. Finally, dogs play a crucial role in both infections as competent reservoir hosts for *A. platys*, carriers of infected ticks to close contact to humans and effective sentinels to assess the risk of *A. phagocytophilum* human infection.

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CHAPTER II

SCIENTIFIC AIMS

The Anaplasmataceae family includes some of the most important pathogens to both dogs and humans and some of them have been identified from ticks and dogs in North Africa. Moreover, both *I. ricinus* and *R. sanguineus* that transmit *A. phagocytophilum* and probably *A. platys*, respectively are present in Morocco. *Anaplasma phagocytophilum* has an extended distribution through the Northern Hemispher. The disease caused by this bacterium, i.e., granulocytic anaplasmosis, is zoonotic and both the prevalence and incidence have dramatically increased in dogs and humans in the USA the past decades. Human cases have also been described in Europe and China, with high mortality rates in the latest country. In addition, life-threatening complications associated with high hospitalization rates and transmission by blood transfusion have been reported to occur in human patients. Clinical signs and laboratory modifications are unspecific resembling other tick-borne diseases and diagnosis can be very challenging for both canine and human patients. *Anaplasma platys* is another widespread tick-borne pathogen, causing infectious cyclic thrombocytopenia in dogs, with non-specific clinical signs resembling those induced by *A. phagocytophilum* infection. Although considered as a pathogen specific of canine species for decades, this bacterium has been shown to infect other animal species and human, highlighting its zoonotic potential.

In Morocco, canine ownership has increased in the past years. In addition, stray dogs are still a major problem in the transmission of some zoonotic diseases such as rabies and leishmaniasis. Despite heavy tick infestation is very frequent even in Moroccan pet dogs and especially in rural areas, ectoparasites prevention is not regularly administered with only a few molecules commercialized, and very limited diagnostic tools of vector-borne diseases (VBDs) available. Although ticks are abundant in Morocco, no data are currently published on tick-borne infections in dogs such as *A. phagocytophilum* and *A. platys*. Therefore, epidemiological studies are crucial to determine if both bacteria are present in both canine and human populations Morocco.

The scientific aims of this study are:

1. To assess canine exposure to selected vector-borne pathogens in Morocco and to determine whether dogs are exposed more specifically to *Anaplama* spp.
2. To evaluate the occurrence of *A. phagocytophilum* and *A. platys* in dogs in Morocco.
3. To evaluate human exposure to *A. phagocytophilum* in Morocco.

CHAPTER III

EXPOSURE TO SELECTED VECTOR-BORNE PATHOGENS IN DOGS IN MOROCCO

DETECTION OF *ANAPLASMA* SPP. AND *EHRLICHIA* SPP. ANTIBODIES, AND *DIROFILA IMMITIS* ANTIGENS IN DOGS FROM SEVEN LOCATIONS OF MOROCCO

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Summary

In Morocco no data has been published on canine exposure to *Anaplasma* spp., *Borrrelia burgdorferi*, and *Ehrlichia* spp., and only one report is available on the occurrence of *Dirofilaria immitis* in dogs. Therefore, the aim of this study was to collect current data on the canine exposure to these vector-borne pathogens (VBPs) in Morocco.

A total of 217 urban (n = 57), rural (n = 110) and military (n = 50) dogs from seven Moroccan locations were screened for *Anaplasma* spp., *B. burgdorferi* and *Ehrlichia* spp. antibodies and for *D. immitis* antigens using a commercial in-clinic ELISA test. Of these dogs, 182 (83.9%) tested positive for at least one pathogen and positivity to two or three pathogens was found in 14.3% and 2.3% of the dogs, respectively.

Ehrlichia spp. antibodies (34.6%) were the most frequently detected followed by *Anaplasma* spp. antibodies (16.6%) and D. immitis antigens (16.1%). None of the dogs was tested seropositive to *B. burgdorferi.* Statistically significant differences in seropositivity rates were found for *Ehrlichia* spp. and *D. immitis* in rural dogs especially those from the north central region ($p < 0.001$) but not for *Anaplasma* spp. No significant difference was found according to the health status of the dog.

This study demonstrates that Moroccan dogs are at high risk of acquiring a vectorborne infection.

Introduction

Canine vector-borne pathogens (VBPs) have been of increasing interest during the past decades because of their increased frequency and their threat to both canine and human health. *Anaplasma phagocytophilum*, *Borrelia burgdorferi*, *Ehrlichia canis* and *Dirofilaria immitis* are among the most important canine VBPs.[1] *Anaplasma phagocytophilum*, *B. burgdorferi* and *D. immitis* are recognized as zoonotic pathogens[1] while *E. canis* could have a zoonotic potential as human infection has been reported.[2] Dogs can play an important epidemiological role in some zoonotic VBPs as competent reservoir hosts, carriers of infected vectors in close contact to humans or effective sentinels to assess the risk for human infection.[1] Therefore, prevalence data in canine species can provide important information concerning the incidence, risk factors, source of exposure, and real-time risk of exposure for human infection. This information, gathered from a particular region is crucial for clinical diagnosis and for effective animal and public health interventions.[3] Due to the complexity of vector-borne diseases (VBDs) diagnosis and control, as well as the possibility of subclinical infection in dogs that increases the risk of disease transmission,[1] epidemiological data aimed to improve knowledge within a region is fundamental.

In North Africa, only a few studies on *A. phagocytophilum*, *B. burgdorferi*, *E. canis* and *D. immitis* exposure and/or infection in dogs have been published[4] and data on these infections is lacking in Morocco. Therefore, the aim of this study was to collect current data on the occurrence of *Anaplasma* spp., *Ehrlichia* spp., *B. burgdorferi* and *D. immitis* exposure in dogs in Morocco using a commercial in-clinic ELISA test.

Materials and methods

Study population

From January 2014 to May 2015, urban and rural client-owned dogs and military dogs were sampled from seven locations of Morocco (Figure 1). Dogs sampled in Benslimane, Tangier, Oujda and Sahara were military dogs; those sampled in Sidi Kacem and Marrakech lived in rural areas and dogs from Rabat were urban client-owned dogs. Military and rural dogs were considered at high risk for acquiring VBPs because of their regular outdoor activities or permanent outdoor living, respectively, and their close contact with other domestic or feral animals. Clinical signs compatible with a tick-borne disease (TBD) (i.e., fever, inappetence or anorexia, lethargy or lameness without orthopedic origin) or heartworm disease (i.e., chronic exercise intolerance, weight loss and coughing) were recorded.

The study protocol was approved by the Ethical Committee for Biomedical Research of the Mohammed V University of Rabat (n°698; July 10, 2014) and the Ministry of Health of Morocco (n°965; June 12, 2014).

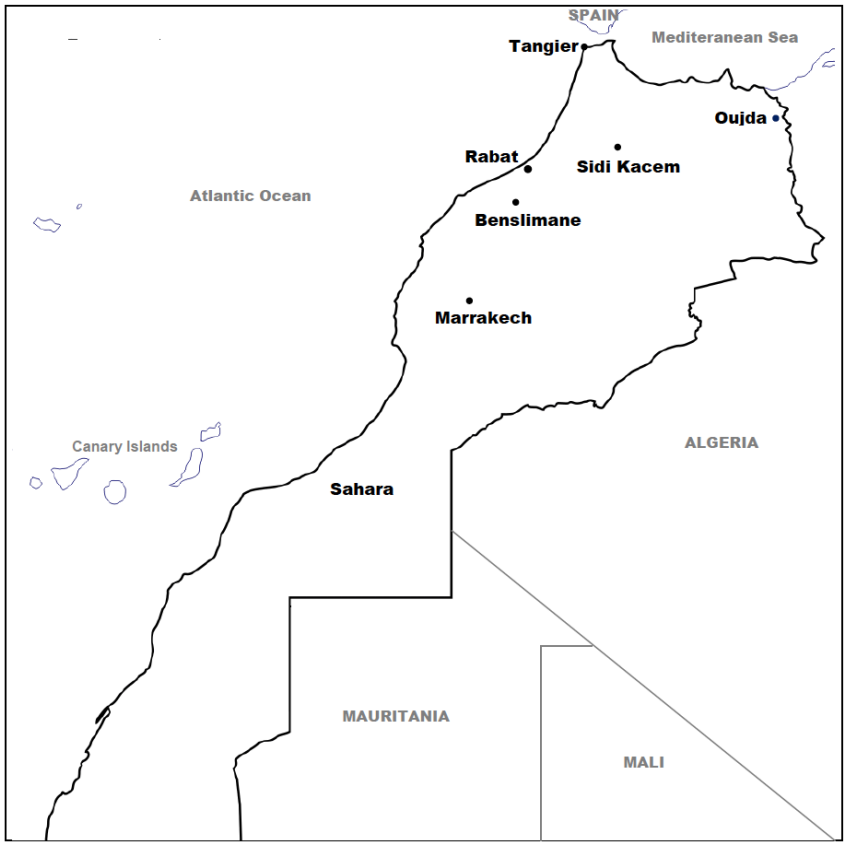

Figure 1. Map of Morocco showing the seven locations of sampling

Blood testing

For each dog, 8 ml of anticoagulated blood was collected and all samples were tested using an in-clinic enzyme-linked immunosorbent assay (ELISA) SNAP 4Dx Plus (IDEXX Laboratories, Inc., Westbrook, ME), according to the manufacturer's directions. The test is registered for the detection of *D. immitis* antigens, and specific antibodies against *Anaplasma phagocytophilum*/*A. platys*, *Ehrlichia canis*, *Ehrlichia ewingii* and *B. burgdorferi* in canine serum, plasma or anticoagulated whole blood. The sensitivity and specificity of the performed test were, respectively, 93.2% and 99.2% for *A. phagocytophilum*, 89.2% and 99.2% for *A. platys*, 96.7% and 98.8% for *B. burgdorferi* sensu lato, 97.8% and 92.3% for *E. canis*, and 98.9% and 99.3% for *D. immitis*, respectively.[5]

Statistical analysis

The statistical analysis was performed using SAS version 6.4 (SAS Institute Inc., Car, NC, USA). The exact logistic regression model was fitted to compare seroreactivity rates between regions, between rural, military and urban dogs and between sick and healthy dogs. The tests were performed at the 5% significance level.

Results and discussion

The dogs sampled included 57 urban, 110 rural and 50 military dogs. Age was available for 137 dogs and ranged from 3 months to 13 years old (mean age = 4.4 years old). Sex and breed were available only for 54 dogs from the western region and included German Shepherds (n = 27), Belgian Shepherds (n = 6), Retrievers (n =6), Pointers (n = 4), Mixte breed dogs (n = 5) and one dog each of Damatian, Rottweiler, Akita Inu, English Setter and Poodle. In the same group, males were more frequently sampled (n = 42) than females (n = 12). The majority of the dogs sampled were apparently healthy (n = 163) and 54 displayed clinical signs compatible with a TBD or heartworm disease.

A total of 182 (83.9%) were positive for at least one pathogen. Table 1 summarizes the results of *Anaplasma* spp. and *Ehlichia* spp. exposure and *D. immitis* infection in dogs for the seven locations. These results are the first describing *Anaplasma* spp. and *Ehrlichia* spp. exposure in dogs in Morocco. The overall positivity rate to *D. immitis* antigens found in our study (16.1%) is quite similar to the prevalence found in a previous study in Rabat (12.3%).[6] However, our positivity rate in Rabat is lower probably because our dogs from this city were client-owned urban dogs rather than stray or rural dogs as in the previous study, or to differences in *D. immitis* detection methods. Prevalence rates of canine *D. immitis* infection up to 17.6% have been recorded in other African and Mediterranean countries.[7-9] None of the dogs tested seropositive to *B. burgdorferi*. Our results contrast with those published in Algeria where antibodies against *A. phagocytophilum* were the most prevalent (47.7%), followed by *B. burgdorferi* (37.6%) and *E. canis* (30.0%).[4] These discrepancies could be due to differences in inclusion criteria and in ticks populations and density between countries or between regions within the same country. Indeed, *B. burgdorferi* is transmitted by *Ixodes* spp. ticks and the main vector in Europe is *I. ricinus*.[4] This tick species has been identified in eastern Morocco and more specifically in the region of Taza, close to the Algerian boundaries,[10-12] but none of the dogs included in our study was sampled in this region. Therefore, the negative result for *B. burgdorferi* antibodies could be due to a selection bias. Table 2 summarizes the simultaneous exposure to two and three VBPs. Co-exposure was found in 14.3% of dogs and was more frequent in rural dogs (26.4%) (Table 3) especially those from the north central region (34.6%) (Table 2).

Table 1. Distribution of dogs positive to *Anaplasma* spp. and *Ehrlichia* spp. antibodies, *D. immitis* antigens and co-infections according to cities and regions.

Region/city	*Anaplasma* spp. (%)		*Ehrlichia* spp. (%)		*D. immitis* (%)		Co-infections (%)	
	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative
Northern region (n=9)	**1 (11.1)**	**8 (88.9)**	**1 (11.1)**	**8 (88.9)**	**1 (11.1)**	**8 (88.9)**	**1 (11.1)**	**8 (88.9)**
Tangier (n=4)	1 (25.0)	3 (75.0)	1 (25.0)	3 (75.0)	0 (0.0)	4 (100.0)	1 (25.0)	3 (75.0)
Oujda (n=5)	0 (0.0)	5 (100.0)	0 (0.0)	5 (100.0)	1 (20.0)	4 (80.0)	0 (0.0)	5 (100.0)
Central northern region (n=78)	**16 (20.5)[a]**	**62 (79.5)**	**47 (60.3)[a]**	**31 (39.7)**	**28 (35.9)[a]**	**50 (64.1)**	**27 (34.6)**	**51 (65.4)**
Sidi Kacem (n=78)	16 (20.5)	62 (79.5)	47 (60.3)	31 (39.7)	28 (35.9)	50 (64.1)	27 (34.6)	51 (65.4)
Northwestern region (n=82)	**16 (19.5)[a]**	**66 (80.5)**	**19 (23.2)[a]**	**63 (76.8)**	**6 (7.3)[a]**	**76 (92.7)**	**8 (9.8)**	**74 (90.2)**
Rabat (n=57)	10 (17.5)	47 (82.5)	10 (17.5)	47 (82.5)	1 (1.7)	56 (98.2)	4 (7.0)	53 (93.0)
Benslimane (n=25)	6 (24.0)	19 (76.0)	9 (36.0)	16 (64.0)	5 (20.0)	20 (80.0)	4 (16.0)	21 (84.0)
Southern region (n=48)	**3 (6.2)[b]**	**45 (93.8)**	**8 (16.7)[b]**	**40 (83.3)**	**0 (0.0)[b]**	**48 (100.0)**	**0 (0.0)**	**48 (100.0)**
Marrakech (n=32)	1 (3.1)	31 (96.9)	8 (25.0)	24 (75.0)	0 (0.0)	32 (100.0)	0 (0.0)	32 (100.0)
Sahara (n=16)	2 (12.5)	14 (87.5)	0 (0.0)	16 (100.0)	0 (0.0)	16 (100.0)	0 (0.0)	16 (100.0)
Total (n=217)	**36 (16.6)**	**181 (83.4)**	**75 (34.6)**	**142 (65.4)**	**35 (16.1)**	**182 (83.9)**	**36 (16.6)**	**181 (83.4)**
Pvalue	0.062		<0.001		<0.001		-	

The Pvalue refers to the diference between regions (excluding Northern regions as only few observatins were available). Regions means with different letters differ significantly at the 5% significance level. The bold values represent the regions.

Table 2. Distribution of dogs positive to two or three pathogens (i.e., *Anaplasma* spp., *Ehrlichia* spp. and *D. immitis*) according to cities and regions.

Co-infections	*Anaplasma*-*Ehrlichia* (%)	*Anaplasma*-*D. immitis* (%)	*Ehrlichia*-*D. immitis* (%)	*Anaplasma*-*Ehrlichia*-*D. immitis* (%)
Northern region (n=9)	**1 (11.1)**	**0 (0.0)**	**0 (0.0)**	**0 (0.0)**
Tangier (n=4)	1 (25.0)	0 (0.0)	0 (0.0)	0 (0.0)
Oujda (n=5)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Central northern region (n=78)	**6 (7.7)**	**0 (0.0)**	**16 (20.5)**	**5 (6.4)**
Sidi Kacem (n=78)	6 (7.7)	0 (0.0)	16 (20.5)	5 (6.4)
Northwestern region (n=82)	**6 (7.3)**	**1 (1.2)**	**1 (1.2)**	**0 (0.0)**
Rabat (n=57)	4 (7.0)	0 (0.0)	0 (0.0)	0 (0.0)
Benslimane (n=25)	2 (8.0)	1 (4.0)	1 (4.0)	0 (0.0)
Southern region (n=48)	**0 (0.0)**	**0 (0.0)**	**0 (0.0)**	**0 (0.0)**
Total (n=217)	**13 (6.0)**	**1 (0.5)**	**17 (7.8)**	**5 (2.3)**

The bold values represent the regions.

Table 3. Distribution of dogs positive to *Anaplasma* spp. and *Ehrlichia* spp. antibodies, *D. immitis* antigens and co-infections according to the health status and living conditions (i.e., rural, urban or military dogs).

Groups	***Anaplasma* spp. (%)**	***Ehrlichia* spp. (%)**	***D. immitis* (%)**	**Co-infections (%)**
Healthy dogs (n=163)	**25 (15.3)**	**57 (35.0)**	**30 (18.4)**	**29 (17.8)**
Rural dogs (n=106)	17 (16.0)	54 (50.9)	28 (26.4)	28 (26.4)
Urban dogs (n=32)	5 (15.6)	2 (6.2)	1 (3.1)	0 (0.0)
Military dogs (n=25)	3 (12.0)	1 (4.0)	1 (4.0)	1 (4.0)
Pvalue	0.93	<0.0001	0.02	-
Sick dogs (n=54)	**11 (20.4)**	**18 (33.3)**	**5 (9.3)**	**8 (14.8)**

The Pvalue refers to differences between dog origin within the healthy dogs group. The bold characters are for healthy and sick groups.

No significant differences in the positivity rates to *Anaplasma* spp. (p = 0.5484), *Ehrlichia* spp. (p = 0.3119) and *D. immitis* (p = 0.2891) was detected according to the age of dogs. The seropositivity rates were significantly higher in the north central region for both *Ehrlichia* spp. and *D. immitis* in comparison to the other regions, but not for *Anaplasma* spp. (Table 1). Similarly, seropositivity rates in rural dogs were significantly higher when compared to military and urban dogs for *Ehrlichia* spp. and *D. immitis* but not for *Anaplasma* spp. (Table 3). This difference could be explained by the statistically significant difference found between regions with the highest prevalence rates recorded in the central northern region where all dogs sampled were rural dogs. Other studies showed higher positivity rates for VBPs in stray and rural dogs,[4,8,9] probably because outdoor living increases the risk of contact with infected vectors. No statistically significant differences were found in the seropositivity rates between dogs displaying clinical signs compatible with a TBD or heartworm and those apparently healthy for *Anaplasma* spp. (p = 0.4025), *Ehrlichia* spp. (p = 0.8702) and *D. immitis* (p = 0.1372). Some reports described significant differences in seropositivity to E. canis according to the health status of the dog and found positive correlation between seropositivity to this bacterium and the presence of clinical signs[4,8] while no correlation with the seropositivity to *A. phagocytophilum* or *D. immitis* was detected.[8]

Serological-based surveys on *Anaplasma* spp. and *Ehrlichia* spp. have two main limitations. The first is that a positive result can be indicative of either an ongoing infection or a previous exposure to the pathogen.[13] The second limitation is the existence of cross-reaction between *Ehrlichia* species (i.e., *E. canis*, *E. ewingii* and *E. chaffeensis*) and between *Anaplasma* species (i.e., *A. phagocytophilum* and *A. platys*).[5] *Ehrlichia chaffeensis* and *E. ewingii* and their respective diseases have been described almost exclusively in some regions of the United States where *Amblyomma americanum* is the only proven competent vector.[14] Consequently, the positivity rates to *Ehrlichia* spp. obtained in our study are likely due to the presence of *E. canis* antibodies. Its main vector, *Rhipicephalus sanguineus*,[15] is present in Morocco[12] and canine exposure to this bacterium has been reported in North African and Mediterranean countries with seroprevalence rates up to 46%.[4,8,16,17] Similarly, the seropositivity to *Anaplasma* spp. in this study is likely to reflect exposure to *A. platys*. Indeed, this bacterium is also most likely transmitted by *Rhipicephalus sanguineus* and has been described in African countries with prevalence rates up to 80.8%.[15,18,19] Additionnaly, *B. burgdorferi* and *A. phagocytophilum* seem to be transmitted by the same *Ixodes* spp. ticks,[8] hence the lack of detection of *B. burgdorferi* antibodies could indicate that *Anaplasma* spp-seropositive dogs in this study might have been exposed to *A. platys*. Finally, only a few prevalence surveys on *A. phagocytophilum* are available from African countries with prevalence rates up to 4% in Africa and the Mediterranean area.[4,9,16,20-25]

Conclusions

This study demonstrates the canine exposure to *Anaplasma* spp., *Ehrlichia* spp. and *D. immitis* in military, rural and urban dogs in seven Moroccan locations with high prevalence rates. Rural dogs, especially from the north central region, were significantly more exposed to *Ehrlichia* spp. and *D. immitis*. This study also described the occurrence of simultaneous exposure to two and three VBPs with *Ehlichia* spp. and *D. immitis* co-exposure the mot frequently detected. These findings highlight the importance of regular preventive measures against arthropod vectors especially in dogs with free access to the outdoors. Veterinarians need to include these diseases in their differential diagnosis and to recommend the use of regular and adapted prophylactic measures to prevent disease transmission. Finally, this study highights the need for large scale prevalence studies to determine the occurrence of these VBPs in all Moroccan regions and associated risk factors. Molecular-based surveys are also mandatory to identify the *Anaplasma* and *Ehrlichia* species circulating in the canine population in Morocco.

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CHAPTER IV

EVALUATION OF *ANAPLASMA* SPP. EXPOSURE AND INFECTION IN DOGS AND HUMANS IN MOROCCO

ANAPLASMA SPP. IN DOGS AND OWNERS IN NORTHWESTERN MOROCCO

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Summary

Anaplasma phagocytophilum is an emerging tick-borne zoonotic pathogen of increased interest worldwide which has been detected in northern Africa. *Anaplasma platys* is also present in this region and could possibly have a zoonotic potential. However, only one recent article reports on the human exposure to *A. phagocytophilum* in Morocco and no data are available on canine exposure to both bacteria. Therefore, we conducted a cross-sectional epidemiological study aiming to assess both canine and human exposure to *Anaplasma* spp. in Morocco. A total of 425 dogs (95 urban, 160 rural and 175 working dogs) and 11 dog owners were sampled from four cities of Morocco. Canine blood samples were screened for *Anaplasma* spp. antibodies by an enzyme-linked immunosorbent assay (ELISA) and for *A. phagocytophilum* and *A. platys* DNA by a real-time polymerase chain reaction (RT-PCR) targeting the *msp2* and *groEL* genes, respectively. Human sera were tested for specific *A. phagocytophilum* immunoglobulin G (IgG) using a commercial immunofluorescence assay (IFA) kit.

Anaplasma spp. antibodies and *A. platys* DNA were detected in 21.9 and 7.5% of the dogs, respectively. *Anaplasma phagocytophilum* DNA was not amplified. *Anaplasma platys* DNA was significantly more frequently amplified for working dogs. No statistically significant differences in the prevalence of *Anaplasma* spp. antibodies or *A. platys* DNA detection were observed between sexes, age classes or in relation to exposure to ticks. A total of 348 *Rhipicephalus sanguineus* (sensu lato) ticks were removed from 35 urban and working dogs. The majority of dog owners (7/10) were seroreactive to *A. phagoyctophilum* IgG (one sample was excluded because of hemolysis).

This study demonstrates the occurrence of *Anaplasma* spp. exposure and *A. platys* infection in dogs, and *A. phagocytophilum* exposure in humans in Morocco.

Introduction

Ticks are considered to transmit the widest number of pathogens when compared to other arthropod vectors, and several of these pathogens are of veterinary and medical importance.[1] Some tick-borne pathogens (TBPs) are considered to be emerging because of several factors that play a crucial role in ticks multiplication and expansion, increasing the likelihood of ticks feeding on humans and animal and transmitting pathogens.[2] Among these emerging TBPs of zoonotic relevance, *Anaplasma phagocytophilum* (formerly *Ehrlichia equi*, *Ehrlichia phagocytophila*, and the human granulocytic ehrlichiosis agent) is an obligate intracellular gram negative bacterium belonging to the family of Anaplasmataceae.[3] This bacterium causes a widespread disease called granulocytic anaplasmosis and is commonly transmitted by *Ixodes* tick species.[4] In the past decades, both human and animal exposure to *A. phagocytophilum* has continuously increased in the USA, Europe and some Asian countries.[4-8] The clinical presentation of human granulocytic anaplasmosis is a non-specific flu-like disease potentially fatal with severe complications, high hospitalization rates and difficult diagnosis.[7-9] Dogs are mostly recognized as incidental hosts and their role as potential reservoir hosts for *A. phagocytophilum* infection is still controversial.[10] However, some authors suggested that dogs may be considered as potential reservoir hosts for *A. phagocytophilum* in some regions, especially in urban environments,[11-14] or at least as effective sentinels to assess the risk for human infection.[15]

Anaplasma platys is another species of *Anaplasma* known to infect dogs, which are considered the main reservoir hosts. This bacterium is most likely transmitted by *Rhipicephalus sanguineus* (s.l.) ticks and is responsible for infectious canine cyclic thrombocytopenia.[16] *Anaplasma platys* is not considered as zoonotic although infection of other domestic animals[17-22] and humans[23-27] have been reported. Both *A. platys* and *A. phagocytophilum* infections remain usually asymptomatic or subclinical in dogs. When present, clinical signs are unspecific and include fever, lethargy, anorexia, lymphadenopathy, lameness, thrombocytopenia and anemia.[15,16]

In Morocco, both *Ixodes ricinus* and *R. sanguineus* (s.l.) ticks are present.[28-30] In addition, *A. phagocytophilum* and *A. platys* were reported in domestic animals and ticks in North Africa.[31-36] However, only one recent report described human exposure to *A. phagocytophilum* in Morroco[37] and no data are available on the canine exposure to both *A. phagocytophilum* and *A. platys*. Therefore, the aim of this study was to assess the occurrence of *Anaplasma* spp. infection and/or exposure in different groups of dogs and dog owners in Morocco.

Material and methods

Dogs

Between December 2013 and May 2015, 425 dogs were sampled from four Moroccan cities (Figure 1) and divided in 3 groups. The first group (Group I) included 95 clientowned dogs sampled in the Veterinary Teaching Hospital (VTH) of the Institut Agronomique et Vétérinaire Hassan II, Rabat (34°01'31"N, 06°50'10"W). These dogs were clustered in two subgroups: Group Ia included 63 dogs without clinical signs compatible with tick-borne diseases (TBDs) and brought to the VTH for vaccination, surgery or post-surgical follow up, dermatology, cardiology or orthopedic consultations, and Group Ib included 32 dogs with clinical signs compatible with TBDs (fever, inappetence or anorexia, lethargy and lameness without orthopedic origin). For each dog of the first group, an epidemiological questionnaire was completed describing the date of sample collection, age, sex, breed, outdoor activities, ectoparasite prophylaxis, exposure to ticks, travel history outside Morocco during the previous year, vaccination status, presenting complains and physical examination. The second group (Group II) was composed of 160 client-owned dogs from the rural region of Sidi Kacem (34°13'00"N, 5°42'00"W). These dogs behave like stray or roaming dogs because of their outdoor living, close contact with other domestic of feral animals, and low health and or wellness care (absence or irregular vaccination and/or, parasite prevention). Information available on this group included age, sex and breed. The third group (Group III) contained 170 military and gendarmerie working dogs sampled in the first kennel of the Royal Army Forces of Benslimane (33°36'44"N, 7°07'16"W) and the kennel of the Royal Gendarmerie of Temara (33°55'36"N, 6°54'44"W), respectively. Data available on these dogs were age, sex and breed. Groups II and III included apparently healthy dogs considered at high risk for acquiring TBPs because of their regular outdoor activities or permanent outdoor living conditions and irregular ectoparasites prevention. All owners gave their consent for enrollment of their dogs.

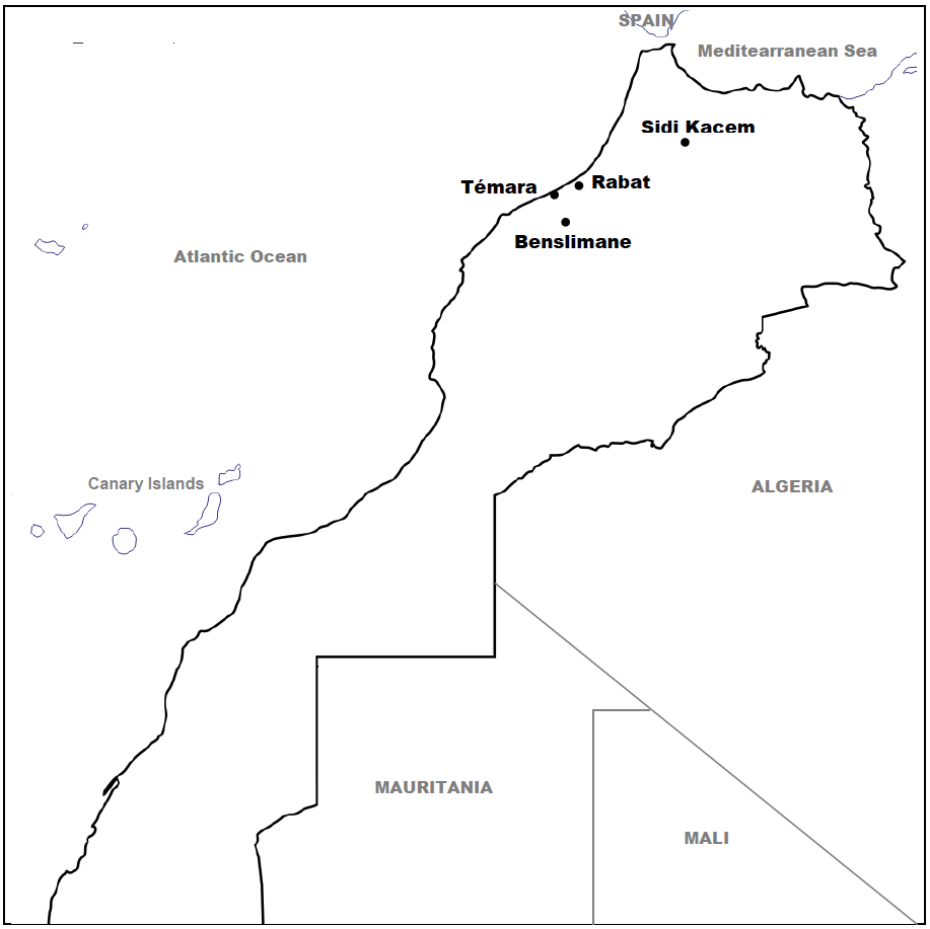

Figure 1. Map of Morocco showing the geographic location of the four cities of sampling

For each dog, 8 ml of non-anticoagulated blood were collected from the cephalic vein. Blood was centrifuged at 3,500 rpm for 10 min and serum was separated, aliquoted and frozen at -32°C. In addition, 2 ml of whole blood collected on ethylenediaminetetra-acetic acid (EDTA) anticoagulant tubes were sampled and frozen at -32°C. The frozen sera and whole blood samples were sent to the IDEXX Laboratories (Sacramento, California, USA) to be tested for for anti-*Anaplasma* spp. antibodies and for *A. phagocytophilum* and *A. platys* using PCR.

Ticks

A total of 348 ticks were removed manually from the dogs included in this study, identified (species, stage, sex)[38] and conserved in 70% ethanol at 4 °C until shipment to the IDEXX Laboratories (Sacramento, California, USA).

Owners

All dog owners of the dogs included in Group I were contacted by phone to be sampled for *A. phagocytophilum* antibodies testing. Only eleven accepted to be enrolled in this study and signed an informed consent forms. An epidemiological report was completed for each owner. Age, city of residence, occupational activity, travels outside Morocco during the previous year, outdoor activities, tick exposure and potential contact with dogs and other domestic animals (cats, horses and ruminants) were recorded.

For each patient, 5 ml of non-anticoagulated blood were collected from the elbow groove vein. Blood samples were centrifuged at 3,500 rpm for 10 min and serum was separated, aliquoted and stored at -32 °C until shipment to the National Reference Laboratory for *A. phagocytophilum* in Queen Astrid Hospital (Brussels, Belgium).

Laboratory procedures

Serological analysis of canine sera (ELISA)

The *Anaplasma* spp. antibody ELISA utilizes orthogonal assay protocols to screen and subsequently confirm the presence of *Anaplasma* antibodies in a serum or plasma sample. The protocols employ microwells coated with *Anaplasma* p44 peptide and *Anaplasma* peptide conjugated to Horseradish peroxydase (HRPO).[39] Briefly, 50 µl of sample was added to a microtiter plate well, followed by 50 µl of conjugate. The plate was incubated for 30 min at room temperature. Wells were washed 5 times with a PBS Tween wash solution, followed by adding 100 µl of TMB substrate and a 15-min incubation step at room temperature. The assay is stopped by adding a stop solution and read at 650 nm using a plate reader spectrophotometer. Positive and negative controls were run in parallel on each plate.

DNA extraction and real-time PCR assays on dogs

EDTA blood samples were used to extract total nucleic acid following a protocol adapted from Boom et al.[40] Briefly, 180 µl whole blood were resuspended in a lysis solution and incubated for 10 min. Lysates were extracted using Whatman binding plates (Thermo Fisher Scientific, Whatham, Massachusetts, USA) on a Corbett X-Tractor platform (Qiagen, Valencia, CA, USA). Nucleic acids were eluted into 150 µl of PCR-grade nuclease-free water (Thermo Fisher Scientific, Whatham,

Massachusetts, USA) and 5 µl amplified in subsequent real-time PCR reactions. Analysis was performed on a Roche LightCycler 480 (Roche Applied Science, Indianapolis, USA) and raw data analyzed using the second derivative maximum method with the 'high sensitivity' setting to generate crossing points (CP values).

Whole blood samples for PCR testing were available only for 362 dogs including 59 from Group Ia, 32 from Group Ib, 104 from Group II and 167 from Group III. *Anaplasma* spp. real-time PCR assays were used from a commercial source (IDEXX Laboratories, Inc., Westbrook, Maine, USA; test code 2824 RealPCRTM test). Real-time PCR tests were designed using a commercially available software (PrimerExpress 3.0) according to the published guidelines.[41] The test was adapted from previous publications[42,43] and consisted of a mixture of two strain specific tests including *A. phagocytophilum* (*msp2* gene, GenBank accession no. DQ519570) and *A. platys* (*groEL* gene, AY848753). PCR tests positive for *Anaplasma* spp. were then screened at the species level using the individual strain specific real-time PCR tests. The internal sample control real-time PCR test was designed using *18S rRNA* (DQ287955). All assays were designed and validated according to industry standards (Thermo Fisher Scientific, Whatham, Massachusetts, USA; User Bulletin #3).

Real-time PCR was run with 6 quality controls including (1) PCR positive controls (quantitatively); (2) PCR negative controls; (3) negative extraction controls; (4) DNA pre-analytical quality control targeting canine *18S rRNA* gene complex; (5) environmental contamination monitoring control; and (6) spike-in internal positive control. These controls assessed the functionality of the PCR test protocols for the (1), absence of contamination in the reagents (2) and laboratory (5), absence of crosscontamination during the extraction process (3), quality and integrity of the DNA as a measure of sample quality (4), reverse transcription protocol (5 and 6) and absence of PCR inhibitory substances as a carryover from the sample matrix (6).

Real-time PCR tests were validated analytically and clinically. For the analytical validation, each assay had to pass 6 validation criteria including amplification efficiency, linearity, reproducibility intra-run, reproducibility inter-run, r-square value and signal to noise ratio of the fluorescent signal. Clinical samples were used to repeat standard curves and to confirm PCR positive results by sequencing with outside flanking primers. A total of 4,125 clinical samples were used during the clinical validation of this panel and test results were compared to either alternative PCR test systems or immunofluorescence assay (IFA) methods.

Serological analysis of human sera (IFA)

Human sera were screened for *A. phagocytophilum* immunoglobulin G (IgG) antibodies by a semi-quantitative indirect IFA using a commercial kit (Focus Diagnostics, Cypress, California, USA) containing HL60 cells infected with a human isolate of *A. phagocytophilum* HGE-1 according to the manufacturer's instructions. Briefly, 5 µl of serum were diluted in 315 µl of phosphate-buffer saline (PBS) (0.01 M, pH = 7.2 ± 0.1). The positive IgG control was also diluted in PBS to obtain five dilutions 1:2, 1:4, 1:8, 1:16 and 1:32. Then, 25 µl of diluted sera were added in the slides wells (one well per sample). The first line of the first slide contained the négative IgG control and the five dilutions of the positive IgG control. The slides were incubated in humid chambers between 35.0 and 36.5 °C for 30 min. After the incubation period, the slides were washed with PBS solution followed by distilled water to eliminated non-conjugated serum antibodies. In the second step, 25 µl of conjugate containing human IgG combined with fluorescein were added in each well. The slides were incubated again then washed in the same formerly described conditions. Finally, the slides were dried, coverslipped using mounting medium and observed with ultraviolet light microscopy (×400). The titer was defined as the reciprocal of the highest dilutions of serum with the homogeneously stained cytoplasmic morulae. A serum titer of ≥ 1:64 was considered as positive for *A. phagocytophilum* IgG according to the manufacturer's instructions. Samples that were positive at the first dilution of 1:64 under ultraviolet light microscopy (×400) were then further diluted to 1:128 and those remaining positive at the second dilution were then tittered at 1:256 and 1:512.

Statistical analysis

Statistical analysis was performed using SAS version 6.4 (SAS Institute Inc., Car, NC, USA). The exact logistic regression model was fitted to compare seroreactivity and PCR positive rates between the different groups, age classes, sex and in relation to the presence of ticks. First, global hypothesis tests were performed, comparing all dog groups, based on the likelihood ratio test (LRT). With an overall significant test, groups were compared pairwise using Bonferroni's multiple comparisons technique at a global significance level of 5%. Significant pairwise comparisons were summarized in terms of the odds ratio (OR) with a 95% confidence interval (95% CI). Other risk factors (sex, tick exposure, age groups) were analysed in the same way.

Results

Serological and molecular screening of dogs

Off the 425 dogs, breed, sex and age were available for 299 (70.3%), 398 (93.6%) and 402 (94.6%) dogs, respectively. Dogs belonged to 23 different breeds with German and Belgian Shepherds (n = 122), Retrievers (n = 58), Saluki (n = 36), Cocker and English Spaniel (n = 27), mixed breeds (n = 19) and Pointers dogs (n = 10) the most frequently found during sampling. Other breds included Poodles (n = 4), Rottweilers (n = 3), Pekingese (n = 3), Aidi (n = 2), Border Collie (n = 2), Pitbull (n = 2), Setters dogs (n = 2) and one dog for Drahthar, Saint Hubert, German Mastiff, Argentin dogo, Dalmatian, Akita Inu, Husky, Havanese and Chihuahua. The age of dogs ranged from 3 months to 14 years-old (mean age 3.2 years-old) and males (n = 257) were more frequently sampled than females (n = 141). Previous ticks bites were available for 226 dogs (53.2%) from Group I (n = 40) and Group III (n = 18).

Table 1 summarises the results of *Anaplasma* spp. antibodies and *A. platys* DNA detection in the three groups of dogs. There were significant differences between dog groups ($\chi2$ = 10.28, df = 3, P = 0.016). Group Ia differed significantly from Group II (OR = 0.32, 95% CI: 0.14-0.75, P = 0.009). None of the 362 dogs screened for *A. phagocytophilum* DNA by PCR was found positive whereas 7.5% (95% CI: 0.05-0.11) of them were positive to *A. platys* (Table 1). There were globally significant differences between dog groups ($\chi2$ = 9.44, df = 3, P = 0.024). The highest prevalence of *A. platys* DNA detection was found in Group III but none of the pairwise comparisons was significant (Table 1). Table 2 summarizes the prevalence of positivity rates to *Anaplasma* spp. antibodies and *A. platys* DNA detection according to sex, age and exposure to ticks. No statistically significant differences were found in seropositivity rates for the sex ($\chi2$ = 2.161, df= 1, P= 0.142), the age groups ($\chi2$ = 1.75, df= 2, P = 0.416) and exposure to ticks ($\chi2$ = 0.83, df = 1, P = 0.363). Similarly, no statistically significant differences were found in positivity rates to *A. platys* DNA detection for sex ($\chi2$ = 2.88, df = 1, P=0.090), the exposure to ticks and age groups ($\chi2$ = 5.05, df = 2, P = 0.080).

Table 1. Number and prevalence (%) of positive and negative dogs to *Anaplasma* spp. antibodies (by ELISA) and *A. platys* DNA detection (by PCR), and positive to both methods in the different groups.

Groups	*Anaplasma* spp. antibodies (%) (n=425)		*A. platys* (%) (n=362)			*Anaplasma spp. and A. platys* (%) (n=362)
	Positive	Negative	Positive	Negative	Not available	
Group I (n=95)	11 (2.6)	84 (19.8)	3 (0.8)	88 (24.3)	4	1 (0.3)
Group Ia (n=63)	7 (1.6)	56 (13.2)	2 (0.5)	57 (15.7)	4	0 (0.0)
Group Ib (n=32)	4 (0.9)	28 (6.6)	1 (0.3)	31 (8.6)	0	1 (0.3)
Group II (n=160)	45 (10.6)	115 (27.1)	4 (1.1)	100 (27.6)	56	1 (0.3)
Group III (n=170)	37 (8.7)	133 (31.3)	20 (5.5)	147 (40.7)	3	9 (2.3)
Total (n=425)	93 (21.9)	332 (78.1)	27 (7.5)	335 (92.5)	63	11 (3.0)

Group I: urban client-owned dogs sample in the VTH; group Ia: urban client-owned dogs sample in the VTH without clinical signs compatible with a TBD; group Ib : urban client-owned dogs sample in the VTH with clinical signs compatible with a TBD; group II: rural client-owned dogs; group III: military and Gendarmerie working dogs.

Table 2. Number and prevalence (%) of positive and negative dogs to *Anaplasma* spp. antibodies (by ELISA) and *A. platys* DNA detection (by PCR) according to the sex, the age and the exposure to ticks.

Variables		*Anaplasma* spp. antibodies (%) (n=425)			*A. platys* DNA (%) (n=362)		
		Positive	Negative	Not available	Positive	Negative	Not available
Sex	Male	59 (13.9)	198 (46.6)	-	20 (5.5)	187 (51.7)	50
	Female	23 (5.4)	118 (27.8)	-	5 (1.4)	123 (34.0)	13
Age (years-old)	<1	9 (2.1)	52 (12.2)	-	3 (0.8)	52 (14.4)	6
	1-5	56 (13.2)	194 (45.6)	-	21 (5.8)	183 (50.6)	46
	≥6	13 (3.0)	61 (14.3)	-	2 (0.5)	62 (17.1)	10
Ticks exposure		40 (9.4)	46 (10.8)	9	40 (11.0)	46 (12.7)	9

Identification of ticks

A total of 348 ticks were removed from 35 dogs and all belonged to *R. sanguineus* (s.l.). Two ticks were nymphs, 284 adult females and 63 adult males. The number of ticks removed from one dog ranged from 1 to 54 (mean number 9.9) (Figure 2). Among the 35 infested dogs, 15 belonged to Group I, 2 to Group II and 18 to Group III. The number of dogs infested by ticks and positive to *Anaplasma* spp. antibodies only, to *A. platys* DNA only or to both tests were eight, three and one, respectively. The only dog infested by ticks and positive for both tests was from Group II.

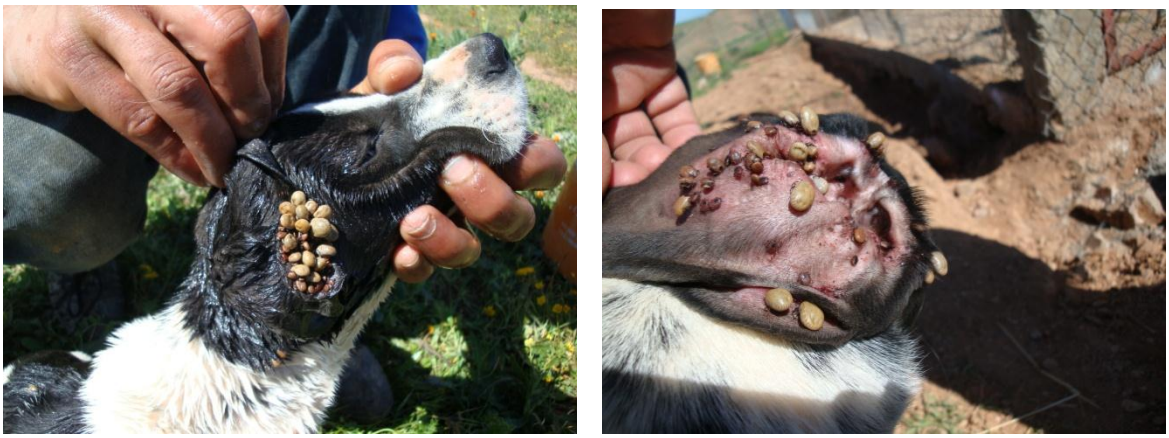

Figure 2. *Rhipicephalus sanguineus* (s.l.) engorged ticks attached to the ear of a dog from group I.

Serological screening of owners

Among the eleven dog owners sampled, three were women and eight were men. Ages ranged from 23 to 66 years, with an average of 51 years. Most lived in Rabat (9/11) and two in surrounding cities (Salé and Arjat). Seven mentioned having leisure outdoor activities in forest or rural areas and one farmer lived in a rural area (Arjat). Five owners reported to have contact with other domestic animals including cats, horses and ruminants. Five owners had additional dogs. Only one owner reported previous exposure to ticks and two traveled to foreign countries during the year.

One sample was excluded due to hemolysis that could interfere with the results according to the manufacturer's instructions. Seven out of the ten remaining sera were positive to *A. phagocytophilum* IgG at the first dilution (1:64) (Figure 3). Among the seropositive owners, three were women and four were men. Four reported regular outdoor activities in the forests of Rabat or the vicinity (Maamora forest, Khémisset, Bouznika and Benslimane). Four owners mentioned to have contact with domestic animals other than dogs. None of the seropositive owners had a travel history outside Morocco during the previous year and two mentioned to be regular blood donors. When further diluted, six, two and one samples remained positive at 1:128, 1:256 and 1:512, respectively (Figure 3). The only sample that remained positive at 1:512 was from a farmer.

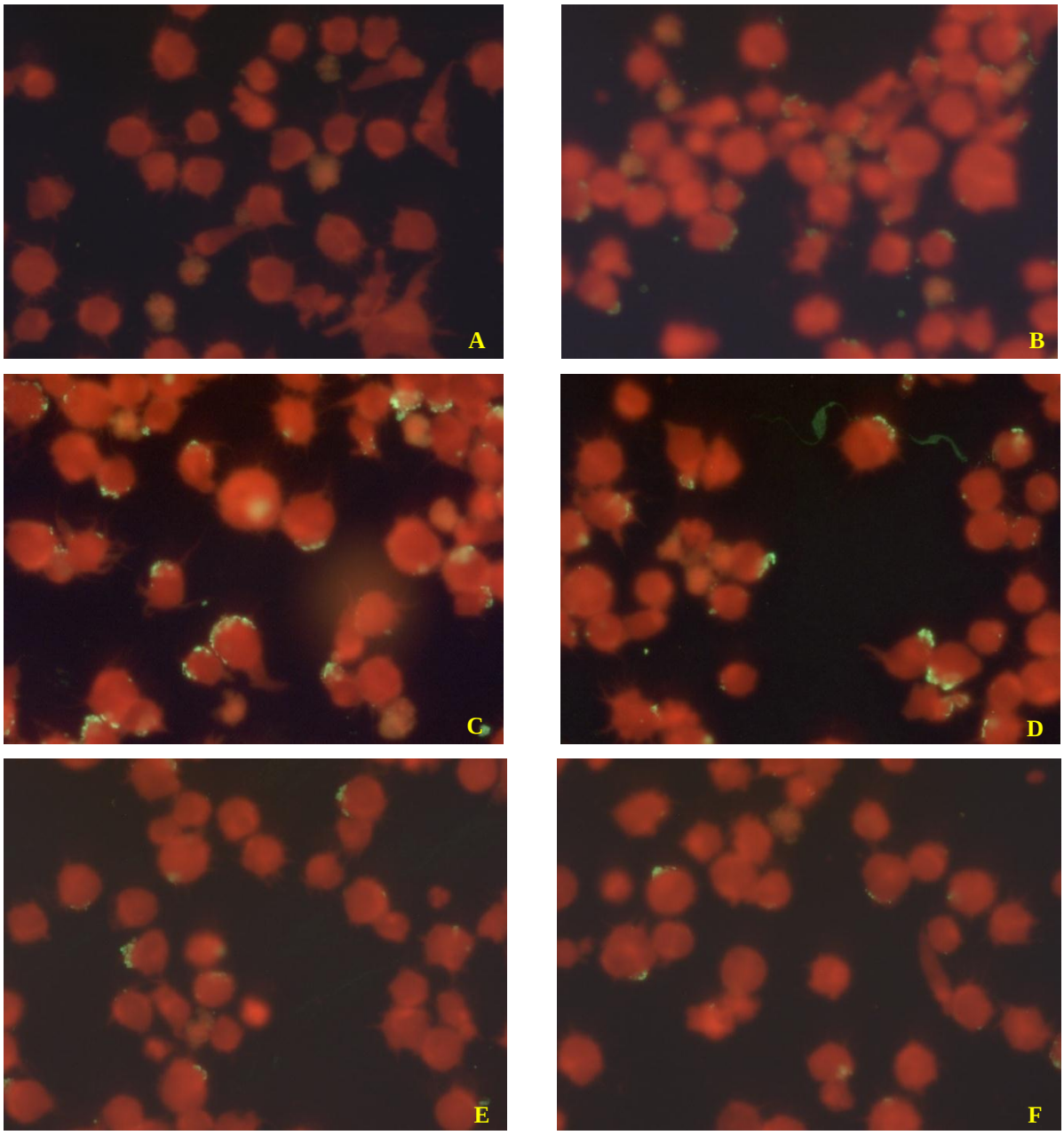

Figure 3. Photographs of ultraviolet light microscopy (×400) of *A. phagocytophilum* IgG semi-quantitative IFA measurement using a commercial kit (Focus Diagnostics, Cypress, California, USA) showing a negative control (A), a positive control (B) and four positive dilutions i.e., 1:64 (C), 1:128 (D); 1:256 (E) and 1:516 (F). The positivity is set on the observation of green morulae surrounding the cell's cytoplasmic membrane.

DISCUSSION

To our knowledge, the results of this cross-sectional study demonstrated for the first time in Morocco a prevalence of 21.9 and 7.5% *of Anaplasma* spp. antibodies and *A. platys* DNA detection in dogs, respectively. It also showed that 7 among 10 dog owners were seroreactive to *A. phagocytophilum* IgG. Currently the two most important Anaplasma species known to infect dogs and humans are *A. platys* and *A. phagocytophilum*.[27] Infection by both species have already been detected in dogs and ticks in North Africa.[28,31,33-36] Our study detected *A. platys* infection in dogs with a prevalence similar to what has been published in Algeria (5.4%).[36] Although not statistically significant, working dogs tested more frequently positive to *A. platys* DNA than rural dogs. Therefore, although considered as a major risk factor for acquiring tick-borne infections,[44-46] outdoor access alone cannot explain the high prevalence in working dogs. Similarly, a study on Senegalese gendarmerie and private kennel living dogs showed a high prevalence of *E. canis* infection,[47] another *R. sanguineus* (s.l.)-transmitted pathogen, probably because this tick species can complete its entire life-cycle either indoor (in houses, kennels and veterinary hospitals where it readily colonizes the infrastructure) or in outdoor environments (peri-urban and rural).[25,47,48] Other factors explaining the higher prevalence in working dogs in our study can be the absence of efficient ectoparasite control programs in this group or the access to areas with higher burdens of *A. platys.*

Our study detected both *Anaplasma* spp. antibodies and *A. platys* DNA in dogs but failed to identify *A. phagocytophilum* DNA. This discrepancy has also been reported in other African, European and American studies.[31,49-51] Cross-reactivity between *Anaplasma* spp. pathogens, especially between *A. phagocytophilum* and *A. platys*, has been reported to occur. Therefore, in regions where both pathogens could co-exist, seropositivity may not enable the distinction at the species level.[16] In areas where *Ixodes* spp. ticks are less prevalent or absent, a positive *Anaplasma* spp. serology could be the result of *A. platys* exposure.[52] Consequently, the fact that we detected exclusively *R. sanguineus* (s.l.) ticks infesting dogs can be supportive of the potential predominance of *A. platys* in Morocco. However, *Ixodes ricinus* ticks are also present in this country[28-30] and could have infected these dogs previously. On the other hand, infection with *A. phagocytophilum* in *Rhipicephalus* spp. has also been reported especially in the Mediterranean countries, and these ticks have been suggested as potential competent vectors of this bacterium in this part of the world.[33,53-56] In a study from Jordan, a high prevalence of *A. phagocytophilum* infection (39.5%) was found in dogs and the most abundant tick species removed was *R. sanguineus* (s.l.) (95.1%) followed by two Haemaphysalis species, whereas no *I. ricinus* was collected from these dogs. The authors suggested that the ticks found in their study could be a possible competent vector of the pathogens detected including *A. phagocytophilum*.[57]

Further studies are necessary to evaluate the ability of *Rhipicephalus* ticks in transmitting *A. phagocytophilum*.

In regions where both *A. platys* and *A. phagocytophilum* are present, a PCR-based assay is required to determine which of the two agents is responsible for positive serological test.[16] Nevertheless, false-negative results are reported to occur with PCR, mainly due to low template concentrations,[27,58] the short duration of *A. phagocytophilum* bacteremia in dogs and the variations in the levels of circulating bacteria.[15,58] In addition, selective amplification of the predominant organism can occur in patients coinfected with genetically similar organisms[27,59] such as *A. phagocytophilum* and *A. platys*, which could be the case in our study. As DNA-based diagnostic tool enables the early detection of the infection by *A. phagocytophilum*, the bacteriemia is of short duration and is usually present transiently during the acute phase of the infection,[15,60,61] negative PCR results might be more difficult to interpret in healthy dogs. Therefore, negative PCR results only indicate that the respective nucleic acid sequence was not detected in the sample evaluated under the assay conditions used and should not be interpreted as evidence of absence of infection.[58] In addition, other factors could explain the negative results in our study mainly the likely degradation of the DNA due to the transport conditions from Morocco to the USA and the selected region of sampling. Indeed, our dogs were sampled exclusively from the western part of Morocco but previous studies detected *I. ricinus* ticks in the eastern regions.[28,29] In addition, *Borrelia burgdorferi* (s.l.), that is transmitted by *Ixodes* spp. ticks, was reported in dogs in Algeria,[31] a neighbour country of Morocco, and ticks in north-eastern Morocco,[30] suggesting that these ticks could be more prevalent in eastern regions.

Consistently with our previous report that detected high prevalence rates of *A. phagocytophilum* exposure in humans in northwestern regions of Morocco,[37] the majority of dog owners sampled were found positive to *A. phagocytophilum* IgG. In our previous study, the contact with dogs or other domestic animals was not a risk factor for the seropositivity,[37] suggesting that other factors such as outdoor activities might be incriminated. Indeed, outdoor activities especially related to forests, meadow habitats and grasslands are considered as a major risk factor for acquiring a tick-borne infection due to the increase risk of contact with infected ticks.[62] Another study has found no significant difference in the seroprevalence of *A. phagocytophilum* among owners of seropositive pets and owners without pets, suggesting that dog ownership may not be a risk factor.[63]

Anaplasma platys was known to infect dogs exclusively, and they are are recognized as the main reservoir hosts. However, recent reports described the infection in domestic ruminants, cats and even in humans.[17-27] In addition, human infestation with *R. sanguineus* (s.l.) has also been reported,[47,57,59] suggesting that *A. platys* could be transmitted to humans through the bite of this tick species. Moreover, all human cases infected with *A. platys* had regular contact with dogs and/or reported infestation of their dogs with *R. sanguineus* (s.l.).[25-27] In addition, in two human cases, the *A. platys* sequence was identical to the sequence found in their dog.[27] This is in contrast to our current and previous study that both failed to detect a relationship between contact with dogs and human seropositivity to *A. phagocytophilum* possibly suggesting that humans in Morocco could be more likely to exposed to this bacterium than to *A. platys*. All previously reported cases of human *A. platys* infection were diagnosed by DNA detection or microscopic identification of morulae within platelets[25-27] and hence, the occurrence of immunological response to this bacterium is unknown. Moreover, to the authors' knowledge, the possible occurrence of crossreaction between *A. platys* and *A. phagocytophilum* antibodies has not been evaluated in humans. The IFA based on HL60-cells infected with a human isolate of *A. phagocytophilum*, such as the one used in our study, are considered to be both sensitive[64] and highly specific for the investigation of seroreactivity to this bacterium[9] with a specificity of 100%, according to the manufacturer.

Rhipicephalus sanguineus (s.l.) is the most common tick in the Mediterranean region.[57] It is known to transmit several pathogens including *Rickettsia conorii*, *Babesia canis*, *Hepatozoon canis* and *E. canis* and probably *Bartonella* spp., *Mycoplasma haemocanis* and *A. platys*.[46] This tick has the particularity to be active during almost all the year and to achieve two or more generations per year. Warmer temperature may contribute to increased tick abundance by a more rapid development. Although *R. sanguineus* (s.l.) ticks usually feed on dogs, they can feed on a wide variety of animal species including humans.[48,65] Therefore, due to its high degree of adaptability, *R. sanguineus* (s.l.) represents a major threat not only to dogs, but also to humans. Furthermore, the report of *E. canis* and *A. platys* human infections[23-27,66,67] emphasizes the importance of *R. sanguineus* (s.l.) and the zoonotic potential of these two infections, and further investigation should be carried out to assess the public health implication.[48]

The major limitations of this study are the restricted area of sampling, the absence of PCR performed on the ticks sampled from dogs, and the small number of owners and dogs with clinical signs compatible with a TBD. Unfortunately, DNA from the ticks collected was too degraded to perform PCR analysis, most probably due to the shipping conditions from Morocco to the USA.

Conclusion

This study demonstrates the *Anaplasma* spp. exposure in humans and dogs in Morocco. To our knowledge, it is also the first report on the occurrence of *A. platys* infection in dogs. Our results showed that working dogs living in kennels are at an increased risk for acquiring this infection. These findings highlight the importance of regular preventive measures against arthropod vectors especially in dogs living in kennels and dogs that have access to outdoor environments. This study also suggets that human exposure to *A. phagocytophilum* is likely to be frequent and emphazises the need for large-scale serological and clinical surveys to better estimate the prevalence of this bacterium and to determine its ability in causing disease in Morocco. Since the human infection by *A. platys* has been reported, Moroccan dogs are frequently infected with this bacterium and dogs are the main reservoir hosts, it is important to evaluate if this bacterium can cause human disease in Morocco and if the infection is associated with an immunological response. This study should serve as an indicator to Moroccan physicians and veterinarians that *A. phagocytophilum* and *A. platys* exposure and infection are not rare, and it will help raise awareness on the potential occurrence of TBDs more generally in this country. Since we reported results in a limited area of the country and on a very limited number of humans, larger and more represeantative surveys are recommended

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CHAPTER V

EVALUATION OF HUMAN EXPOSURE TO *ANAPLASMA PHAGOCYTOPHILUM* IN NORTHWESTERN MOROCCO

HUMAN EXPOSURE TO *ANAPLASMA PHAGOCYTOPHILUM* IN TWO CITIES OF NOTHWESTERN MOROCCO

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Summary

Anaplasma phagocytophilum is an emerging tick-borne zoonotic bacterium with extensive increased interest. Epidemiological data are available in several regions of the USA, Europe and Asia in contrast to other parts of the world such as North Africa.

Blood samples of 261 healthy individuals divided in two groups i.e., dog handlers and blood donors were analyzed. Indirect immunofluorescent assay using a commercial kit was performed to detect specific *A. phagocytophilum* IgG. Two dilutions were used to assess the prevalence of seroreactive samples. Demographic variables were assessed as potential risk factors using exact logistic regression.

Seropositivity rates reached 37% and 27% in dog handlers and 36% and 22% in blood donors. No statistically significant differences were found in the prevalence rates between the two groups. Analysis of risk factors such as gender, age groups, outdoor activities, self-reported previous exposure to ticks, or contact with domestic animals (dogs, cats, ruminants and horses) did not shown any significant difference.

Anaplasma phagocytophilum exposure was common in both high-risk population and blood donors in Morocco.

Introduction

Anaplasma phagocytophilum is an obligate intracellular gram-negative bacterium that infects neutrophils. The bacterium causes an emerging zoonotic tick-borne disease (TBD) called granulocytic anaplasmosis,[1] and is mostly transmitted to humans through the bites of ticks of the *Ixodes* genus. However, other modes of transmission have been described including transplacental transmission, percutaneous exposure or inhalation of the contaminated blood of deer, nosocomial infection following direct contact with blood and respiratory secretions and through blood transfusions.[2,3]

Human granulocytic anaplasmosis (HGA) is an unspecific flu-like illness that is typically characterized by the acute onset of fever, headache, chills, myalgia, malaise, nausea, and cough. Depending on several risk factors, which include advanced age, immunosuppression, co-morbidities and delays in the onset of treatment, HGA can be mild or fatal.[4,5] Life-threatening complications occur in 3% of cases. Consequently, half of the HGA cases are hospitalized and up to 17% of patients require admission to intensive care units, especially when the diagnosis and treatment are delayed.[1,4] Therefore, the Infectious Diseases Society of America recommends that antimicrobial therapy be given to every person suspected of having HGA on the basis of their clinical presentation, so as not to delay the treatment.[6] Due to the potentially serious outcome and the difficulty of the diagnosis, epidemiological data on the prevalence and distribution of human cases within a country are important to increase awareness of physicians and to develop adapted public health strategies to prevent and control this disease.[7]

HGA commonly occurs in the USA and Europe, and it is increasingly diagnosed in some Asian countries.[6,8] In the USA, at least 15,952 HGA cases were reported since 1995 and a 12-fold increased incidence has been observed between 2001 and 2011.[4] In China, the exposure to *A. phagocytophilum* has continuously increased from 8.8% to 59.2% in high-risk populations between 2006 and 2009.[3] Despite a moderate to high seroprevalence in several countries, HGA is still unrecognized and rarely diagnosed due to several factors including limited epidemiological information, difficult diagnosis, asymptomatic or subclinical infections and the lack of awareness among physicians and the public.[2,3,6] Moreover, the occurrence of HGA is unknown in many regions of the world such as Oceania, South America, Africa, and in large regions of Asia. To the author's knowledge, no data are available in North Africa on either the occurrence of HGA or the prevalence of human exposure to *A. phagocytophilum*. However, ticks are abundant in this region and might represent a hazard for both animal and human public health.[9] Therefore, we carried out a cross-sectional epidemiological serologic survey to investigate the potential human exposure to *A. phagocytophilum* in Morocco.

Materials and Methods

Study population

Between June and September 2015, 261 healthy individuals from two groups were sampled from three cities of Morocco (Figure 1). The first group included 144 military and police dog handlers from the first kennel of the Royal Forces Army of Benslimane and the kennel of the Royal Gendarmerie of Temara. This group was considered to be at a high risk for TBDs because of their regular contact with dogs and outdoor occupational activities. The data collected on this group included age and exposure to ticks. The second group included 117 blood donors from the Regional Transfusion Centre of Rabat. All of the blood donors were informed on the purpose of the survey and signed informed consent forms before enrollment. An epidemiological report was completed for each blood donor containing data on the age, city of residence (Figure 1), occupation, travels outside Morocco during the previous year, outdoor activities, tick exposure and potential contact with dogs and other domestic animals (i.e., cats, horses and ruminants).

Figure 1. Map of Morocco showing the cities of sampling (in bold) and the cities of residence and of outdoor activities of the blood donors.

The study protocol was approved by the Ethical Committee for Biomedical Research of the Mohammed V University of Rabat (n°698; July 10, 2014) and the Ministry of Health of Morocco (n°965; June 12, 2014).

Blood sampling

For each person included in the study, 5 ml of non-anticoagulated blood was collected from the elbow groove veins. Blood samples were centrifuged at 3500 rpm during 10 min at 15°C and sera were aliquoted and stored at -32°C until shipment to the National Reference Laboratory for *A. phagocytophilum* in Belgium.

Serological tests

Immunoglobulin G (IgG) antibodies were detected against *A. phagocytophilum* by a semi-quantitative indirect immunofluorescent assay (IFA) using a commercial kit (Focus Diagnostics, Cypress, California, USA) containing HL60 cells infected with a human isolate of *A. phagocytophilum* HGE-1 according to the manufacturer's instructions. Briefly, 5 µL of serum were diluted in 315 µL of phosphate-buffer saline (PBS) (0.01 M, pH = 7.2±0.1). The positive IgG control was also diluted into the following five dilutions: 1:2, 1:4, 1:8, 1:16 and 1:32. Then, 25 µL of diluted sera were added in the wells of each slide. The first line of the first slide contained the negative IgG control and the five dilutions of the positive IgG control. The slides were incubated in humid chambers between 35 and 36.5°C for 30 min, then they were washed with the PBS solution followed by distilled water to eliminated non-conjugated serum antibodies. Next, 25 µL of the conjugate containing human IgG and fluorescein were added to each well. The slides were incubated again and washed as described above. Finally, the slides were dried and coverslipped using a mounting medium and were examined under ultraviolet light microscopy (×400). The titer was defined as the reciprocal of the highest dilutions of serum with the homogeneously stained cytoplasmic morulae (Figure 2). A serum titer of ≥ 1:64 was considered as positive for *A. phagocytophilum* IgG, according to the instructions provided by the manufacturer. Samples that were positive at the first dilution of 1:64 were then further diluted to 1:128 and those remaining positive at the second dilution were then tittered at 1:256 and 1:512. Ten samples were reassessed by a blinded technician from the laboratory at a dilution of 1:64 and the results were confirmed in all cases.

Statistical analysis

A statistical analysis was performed using SAS version 6.4 (SAS Institute Inc., Car, NC, USA). The exact logistic regression model was fitted to compare seroreactivity rates between both dog handler and blood donor groups and between gender, the presence or absence of outdoor activities, exposure to ticks, dogs or other domestic animals inside the blood donor group. The statistical significance was set at 5%. The results were summarized in terms of the odds ratio with a 95% confidence interval.

Results

Eight samples were excluded due to hemolysis that could interfere with the results according to the manufacturer instructions. A total of 138 dog handlers (54.5%) and 115 blood donors (45.4%) were included in the study. The majority of blood donors (105/115) lived in Rabat or the surrounding cities whereas nine blood donors were from other cities (Table 1).

The city of origin was unavailable for one blood donor. All dog handlers were men between 21 and 51 years of age (average age: 33 years). The blood donors group included 63 men (54.8%) and 52 women (42.2%), and their ages ranged from 18 to 61 years (average age: 39 years). The distribution according to the epidemiological variables in the two groups is summarized in Table 2.

Outdoor activities in the forest or rural areas were either occupational or for leisure (picnic, hiking, jogging, walking or hunting). More than half of the blood donors (47/86) reported their outdoor activities in the region of Rabat-Salé-Kenitra region and 20.9% (18/86) reported their activities in other Moroccan regions including Moulay Bousselham, Gharb region, Tangier, Ouazzane, Rif mountain, Ifrane, Azrou, Khouribga, Oued Zem, Nador, Taza, Oujda, El Jadida, Safi, Essaouira, Agadir, Tiznit, Dakkhla, Beni Mellal, Azilal, Marrakech, Ait Baha, Zagora, Taroudant and the High Atlas mountains (Figure 1). The remaining 21 blood donors (24.4%) reported their outdoor activities in both Rabat-Salé-Kenitra and other regions. Previous exposure to ticks was recorded in 1.4 (2/138) and 6.1% (7/117) of dog handlers and blood donors, respectively. Travel outside of Morocco was recorded in 15.6% (18/115) of blood donors and ten traveled to two or more countries.

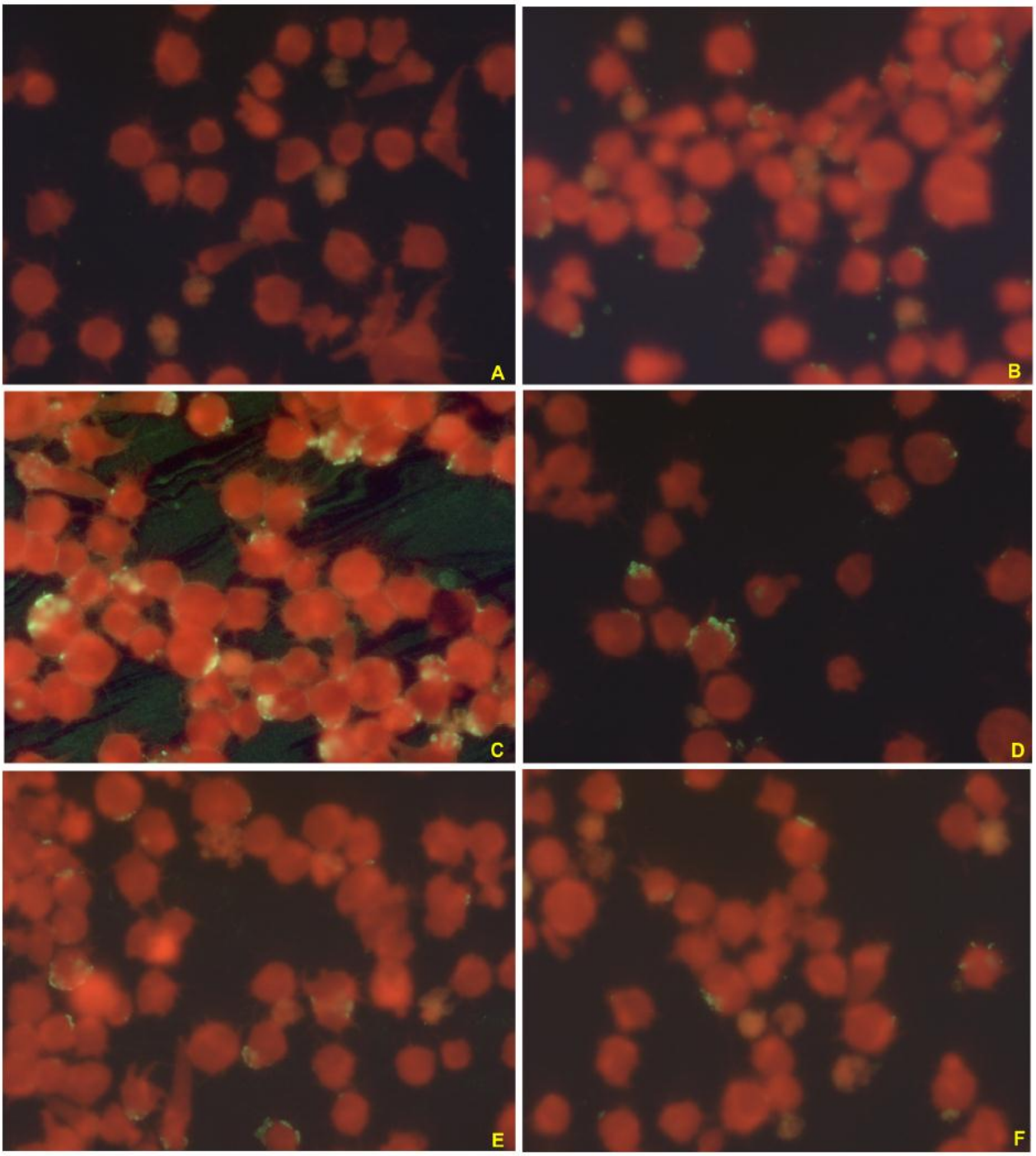

Figure 2. Photographs of ultraviolet light microscopy (×400) of *A. phagocytophilum* IgG semi-quantitative IFA measurement using a commercial kit (Focus Diagnostics, Cypress, California, USA) showing a negative control (A), a positive control (B) and four positive dilutions i.e., 1:64 (C), 1:128 (D), 1:256 (E) and 1:516 (F) from the same patient. The positivity is set on the observation of green morulae surrounding the cell's cytoplasmic membrane.

Table 1. Distribution of the number of blood donors and of positive samples for both dilutions according to city.

Administrative region	City	Distance to Rabat (km)	Number of blood donors	Number of positive IgG 1:64	Number of positive IgG 1:128
Rabat-Salé-Kénitra	Rabat		55	19	10
	Salé	5.2	32	11	6
	Temara	8.0	13	5	2
	Ain El Aouda	30.0	1	1	1
	Sidi Allal El Bahraoui	37.7	1	0	0
	Kenitra	55.0	1	0	0
Casablanca-Settat	Bouznika	39.6	1	1	1
	Benslimane	60.0	1	0	0
Béni Mellal-Khénifra	Khenifra	237.4	1	1	1
	Beni Mellal	233.1	1	1	1
Tanger-Tétouan-Al Hoceima	Tangier	250.0	1	1	1
Souss-Massa-Drâa	Tinghir	477.0	1	0	0
	Agadir	547.0	3	1	1
	Tizi n'Tichka		1	0	0
Guelmim-Oued Noun	Sidi Ifni	686.0	1	0	0

Abbreviations: IgG, immunoglobulin G.

The seropositivity rates for *A. phagocytophilum* IgG at the first dilution reached 37.0% (51/138) and 35.7% (41/115) in dog handlers and blood donors, respectively (Table 3 and Figure 3). At the second dilution, 27.5% (38/138) and 21.7% (25/115) of sera were still reactive in the dog handlers and the blood donors groups, respectively (Table 3 and Figure 3). Most seropositive blood donors for both dilutions (i.e., 1:64 and 1:128) were from the region of Rabat-Salé-Kénitra (Figure 4).

Table 2. Distribution of age, sex, exposure to ticks, contact with dogs or other domestic animals and travel history outside Morocco in both dog handlers (n = 138) and blood donors (n = 115) groups.

Variables		Dog handlers (%)	Blood donors (%)
Sex	Men	138 (100)	63 (54.8)
	Women	0 (0.0)	52 (45.2)
Age (years-old)	≤20	1 (0.7)	7 (6.1)
	21-30	78 (56.5)	26 (22.6)
	31-40	47 (34.1)	27 (23.5)
	41-50	10 (7.2)	36 (31.3)
	>50	1 (0.7)	19 (16.5)
Exposure to ticks		2 (1.4)	7 (6.1)
Outdoor activities		138 (100)	86 (74.8)
Contact with dogs		138 (100)	11 (9.6)
Contact with other domestic animals		-	17 (14.8)
Travel		0 (0.0)	18 (15.7)

Table 3. Number of seropositive samples in both dog handlers (n=138) and blood donors (n=115) groups at the four different dilutions.

Variables	Dog handlers (%)	Blood donors (%)	OR	95%CI	P value
IgG 1:64	51 (37.0)	41 (35.7)	1.05	0.61-1.83	0.90
IgG 1:128	38 (27.5)	25 (21.7)	1.37	0.74-2.56	0.31
IgG 1:256	11 (8.0)	2(1.7)	-	-	-
IgG 1:512	7 (5.1)	2 (1.7)	-	-	-

Abbreviations: IgG, immunoglobulin G; OR, odd ratio; 95%CI, 95% confidence interval.

No statistically significant differences were found between the two groups considering the seroreactivity rates at both dilutions (Table 3). Similarly, no statistically significant differences were found in the blood donor group when comparing between gender, age groups, the presence of outdoor activities, exposure to ticks, and contact with dogs or other domestic animals at both dilutions (Table 2). In the dog handlers group, 11 (8.0%) and 7 (5.1%) of the sera were still positive when further diluted to 1:256 and 1:512, respectively (Table 3 and Figure 3). Only two of the samples remained positive at both 1:256 and 1:512 in the blood donors group (Table 3 and Figure 3).

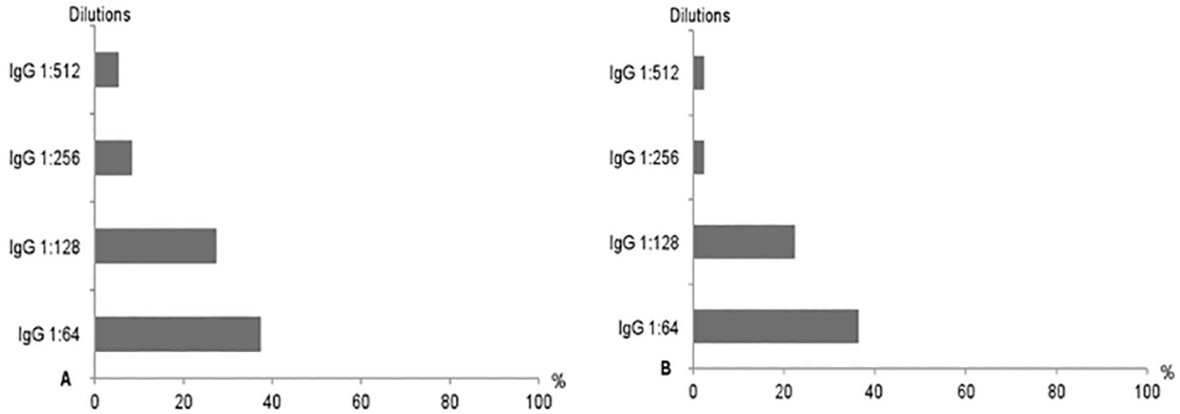

Figure 3. Distribution of positivity rates for the four *A. phagocytophilum* IgG dilutions (i.e, 1:64, 1:128, 1:256 and 1:516) in both dog handlers (A) and blood donors (B) groups.

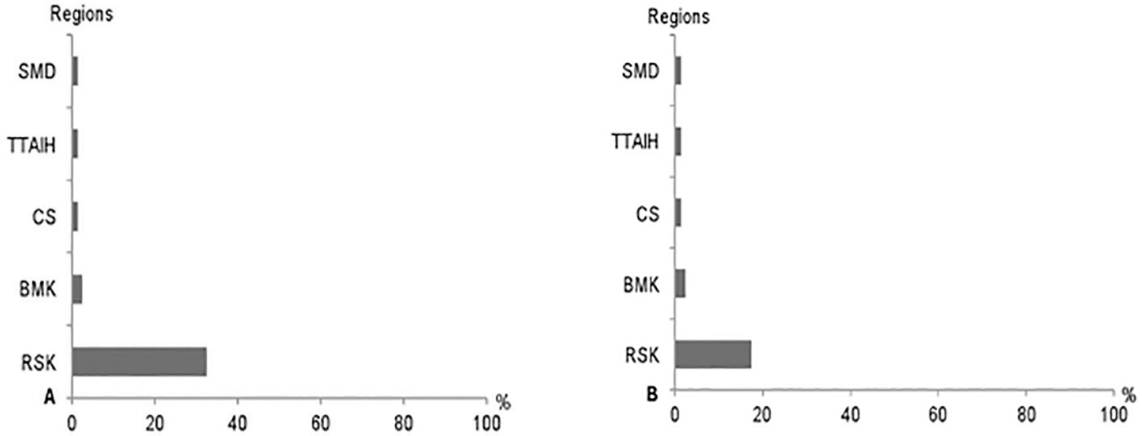

Figure 4. Distribution of *A. phagocytophilum* IgG positivity rates in blood donors according to the region of living in Rabat-Salé-Kénitra (RSK), Casablanca-Settat (CS), Tangier-Tétouane-Al Hoceima (TTAlH) and Souss-Massa-Drâa (SMD) regions and for both 1:64 (A) and 1:128 (B) dilutions.

Discussion

To the author's knowledge, this is the first report investigating human exposure to *A. phagocytophilum* in Africa. In Europe, the USA and Asia, several reports have investigated the prevalence of human exposure in blood donors[10-19] and in high-risk populations including people living in forest areas and forestry workers,[10,12,16,20-25] people living in rural areas and farmers,[3,8,19,26-28] hunters,[8,11] national parks rangers,[29-30] military personnel,[31] people in close contact with domestic animals,[7,21] and people at high risk of exposure or previously exposed to ticks.[7,32,33] The prevalences recorded in high-risk populations or in endemic areas were up to 32%, 35.6%, and 33.7% in Europe,[23] the USA,[34] and China,[35] respectively. However, several serological methods and cutoffs were used, which made the comparison between these studies difficult.[31] When comparing the results of military dog handlers obtained in this study at the threshold of 1:64, i.e., 37%, with other Chinese[24,35] and European[12,21,23,25,28,32,36] reports using the same method and the same cutoff, it appears that the prevalence in Morocco is higher. The highest prevalences recorded in both China and Europe were 20%[7] and 9.6%,[25] respectively. One study from Cyprus reported a prevalence of 32% with a cutoff of 1:128,[23] which is slightly higher than the results found in Morocco at the same cut off (27.5%). When comparing the results of this study with high-risk populations from other European Mediterranean countries such as Italy (8.8%),[22] Portugal (5.4%),[17] and Spain (1.4%),[21] the prevalence found in Moroccan dog handlers is higher at both the first and second dilutions.

Moreover, the prevalence in Moroccan dog handlers is even higher than the prevalence found in patients with clinical signs and history of tick bites in Belgium.[5] Because high-risk populations were shown to have a significantly higher prevalence of *A. phagocytophilum* exposure,[3,16,31] they may not reflect the true exposure of the general population of the same country.[7] Therefore, a more representative sample including blood donors with more diverse social and intellectual levels, occupational and leisure activities would be a better sample to estimate the prevalence of *A. phagocytophilum* exposure in Morocco. In addition, the high seroprevalence rates in blood donors of some geographic locations, the potential asymptomatic or subclinical evolution of the disease, the survival of *A. phagocytophilum* in refrigerated blood products and documented transfusion-transmitted HGA cases, provide further reasons to screen blood donors in Morocco. Although only a few cases of transfusion-transmitted anaplasmosis have been reported, *A. phagocytophilum* infection is among the TBDs that are considered to represent a potential risk for transmission by blood transfusion. In addition, because sharing blood products between different areas is growing, such an acute illness after blood transfusion should be included in the differential diagnosis even in nonendemic areas.[2,37] Our results showed that even in the blood donor group, high prevalences of 35.7% and 21.7% at both the 1:64 and

1:128 dilutions, respectively, were recorded. When compared to European prevalences in blood donors using the same method and the same cutoffs, these results are higher than those published in Poland (2%),[12] and Austria (9%),[15] but they are similar to those from Greece (21.4%).[18] Without taking into account the method and the cutoffs, the results from Moroccan blood donors are even higher than those from several US[13,14] and European reports.[10,11,16,17,19,33,38] In several reports that compared the seroreactivity rates of blood donors to those of high-risk populations, significant differences were found;[10,16,17] these findings are in contrast to our report. These differences could be due to the relatively high proportion of the blood donors that report outdoor activities, which could then increase the possible exposure to ticks and thus predispose them to *A. phagocytophilum* infection.

No risk factors were identified in this survey in either group. Similarly, some reports failed to identify specific demographic variables as potential risk factors.[12,17,23,25,28] In contrast, other reports demonstrated that seropositivity rates were significantly higher in men,[3,23,30] in age groups from 20 to [403,23] and 40 to 65 years of age[32] and the rates increased with age.[29] Seropositivity rates among Moroccan blood donors were higher in men especially for the dilution of 1:128 and lower in the age group ≤20 year-old, although this was not statistically significant. No statistically significant associations between seroreactivity rates and the contact with animals or outdoor activities were found in this study. However, the chance of coming into contact with infected ticks depends on several epidemiological and ecological factors, such as the environment, the presence of appropriate hosts and reservoirs. Consequently, outdoor activities that are especially related to wooded areas, meadow habitats and grasslands are considered to be some of the major risk factors for acquiring TBDs.[39] Moreover, a large number of participants to a study from Germany mentioned contracting their most recent tick bite in their gardens and half of the participants with past exposure to *A. phagocytophilum* listed gardening as a regular leisure activity; despite a comparatively low risk of exposure associated with this activity. Therefore, public health measures to increase awareness for TBDs should also target the large portion of the population who are involved in comparatively low risk outdoor activities such as gardening, cycling or walking.[32] Although not statistically significant, a high proportion (74.8%) of the blood donors mentioned participating in outdoor activities. Consequently, the obvious popularity of outdoor activities may predispose a large number of people to the risk of infection by *A. phagocytophilum*. Only a small portion (3.6%) of the tested population had a history of tick exposure without any significant difference between both groups. Similarly, several surveys did not find any association between selfreported exposure to ticks and the seroreactivity rates of *A. phagocytophilum.*[3,10,12,25,28,40] Moreover, a range of studies demonstrated seropositivity among the blood donors and the control populations without a specific history of a tick bite.[32] Another report described the highest seropositivity among persons who denied having tick bites.[25] A study investigating the risk of acquiring a tick-borne pathogen after a tick

bite failed to identify a significant difference between the group of persons bitten by ticks infected with *A. phagocytophilum* and the group bitten by uninfected ticks.[40] The possible explanations for this oversight could be that the stage of feeding ticks as nymphs and larvae may not be detectable because of their small size or that the capacity of ticks to modulate host immune and inflammatory responses may also decrease the chance of detection.[12,28] Further, several persons from the blood donor group that were questioned about previous contact with ticks were not familiar with these parasites. Therefore, *A. phagocytophilum* infection should not be ruled out in the absence of self-reported previous tick exposure.[4]

Most of the epidemiological surveys about *A. phagocytophilum* have used only the indirect IFA or the enzyme linked immunosorbent assay (ELISA). Either technique used alone with the standard cutoffs may overestimate the prevalence of antibodies.[28,40] The World Health Organization guidelines set the cutoff at the 98th percentile i.e., at 1:128, to fulfil the requirements for seroepidemiological studies. This cutoff should reduce the overestimation of the seroprevalence and therefore provide reliable information with regard to previous infections.[15,18] The overestimation of the seroreactivity with IFA testing might be due to false-positive results secondary to potential cross-reactions.[40] These results can be observed with several other vector-borne pathogens including tick-borne encephalitis virus (6.7%), *Rickettsia conorii* (8%), *Coxiella burnetii* (10%), *Borrelia burgdorferi* (16.7%) and *Bartonella quintana* (70%).[5] The Epstein-Barr virus infection, autoimmune disorders and *Ehrlichia* species may also induce cross-reactivity.[3,15,33,40] However, two studies have failed to demonstrate an increased reactivity to *A. phagocytophilum* in samples that were seropositive to Epstein-Barr virus, cytomegalovirus, parvovirus B19, *Toxoplasma gondii*, *Borrelia burgdorferi* sensu lato, *Coxiella burnettii*, *Rickettsia conorii* and *E. chaffeensis*.[15,21] Moreover, IFA based on HL60-cells infected with a human isolate of *A. phagocytophilum* are considered to be both sensitive[15] and highly specific for the investigation of seroreactivity.[5] According to the manufacturer, the specificity of this test reaches 100%, and the sensitivity depends on the period between the moment of sampling and the beginning of the clinical signs, which ranges from 66.7% to 100%.

Clinical data were not recorded in our study; thus it is unknown whether the subjects who were seropositive to *A. phagocytophilum* experienced any clinical signs before the date of sampling. Although one previous report has found a positive association between fever in the last two years and a high seroprevalence of *A. phagocytophilum*,[3] all or almost all seropositive persons denied any clinical symptoms of HGA in several epidemiological surveys especially in Europe,[18,25] suggesting that a high proportion of the infections could be subclinical.[31] Other possible reasons for the discrepancy between a high seroprevalence and a low incidence of the disease include underdiagnosis or misdiagnosis due to

the unawareness of physicians, the circulation of variants that are non-pathogenic for humans, which may cause only transient infections without the relevant clinical signs and the potential serologic cross-reactivity with other bacteria.[32,40] Despite a low incidence rate and because the severity of the disease is closely linked to delayed diagnosis and treatment, some authors have emphasized the importance of clinicians awareness to promptly diagnose this infection especially in high-risk areas and even in persons without a self-reported history of a tick bite.[28] At least, *A. phagocytophilum* should be considered in the differential diagnosis of flu-like syndromes, febrile patients especially from high-risk areas, febrile illness of unknown etiology or in those who are not responding to beta-lactam antibiotics or macrolides.[16,20,21] Only one serum sample was performed for each participant; samples were not paired and IgM were not measured. Therefore, it was not possible to estimate the incidence of seroconversion and evaluate a potential acute exposure.[28] IgM antibodies are detectable during the first 40 days after infection and IgG seroconversion occurs approximately 20-40 days after the onset of symptoms and persists for several months to years post infection. Therefore, with a single positive IgG titer, it is not possible to distinguish between current and past exposure to *A. phagocytophilum*.[35] In addition, serological testing close to the onset of symptoms is usually negative.[4,6,32] However, IgM testing is reported to be less sensitive than IgG detection, even during early stages of infection.[4] Sampling took place only in two cities of Morocco and subjects' deployment histories were unavailable in the dog handler group. Therefore, no valuable data were available on human exposure in other regions of the country or the distribution or the presence of specific foci within some regions. A more comprehensive and representative study should be conducted to better estimate the prevalence of this bacterium in Morocco.

Conclusion

To the author's knowledge, this study is the first evidence of human exposure to *A. phagocytophilum* or to an antigenically similar bacterium in Morocco. The very high prevalence rate found in both high-risk populations and blood donors indicated the necessity for large-scale serologic surveys to better estimate the prevalence of this bacterium in Morocco. We hope that this study can serve as an indicator to Moroccan physicians that *A. phagocytophilum* infection is present and that this will help raise awareness of the potential occurrence of TBDs. Further studies especially those based on the isolation of the causative agent from patients with clinical signs compatible with HGA are warranted to clearly confirm the presence of the bacterium and to assess its role in causing disease in Morocco. Investigations of the epidemiology and the ecology of the bacterium in Morocco are also needed.

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CHAPTER VI

GENERAL DISCUSSION

1. Prevalence of vector-borne pathogens exposure in dogs in Morocco

Our study demonstrates that dogs in Morocco are frequently exposed to *Anaplasma* spp. (21.9%), *Ehrlichia* spp. (34.6%) and *D. immitis* (16.1%). In addition, this study reported *A. platys* infection in dogs for the first time in Morocco with a prevalence of 7.5%. Before this survey, only a few and sparse data were available on VBPs in dogs in Morocco, other than *Leishmania* spp., and none on TBPs. Only one previous report described canine infection with *D. immitis* in a small number of stray and rural dogs from the region of Rabat[1] and all previous studies on Anaplasmataceae tick-borne pathogens were conducted mainly on ticks.[2-4] Although a few studies demonstrated the occurrence of *A. phagocytophilum*, *A. platys*, *B. burgdorferi*, and *E. canis* in African countries close to Morocco,[5-8] VBPs often have geographical variations in their ecology and prevalence due to differences in host and vector distribution, and to biotic and abiotic influences on the ecology.[9] Although differences in diagnostic methods and studied populations might have an impact on differences found between our study and the results in Algerian dogs,[7] geographical variation in ecological factors are also important to consider. These variations can also influence the spatial distribution and prevalence between regions within a given country.[9,10] Indeed, we demonstrated that rural dogs, especially from the central northern region of Morocco are exposed more frequently to *Ehrlichia* spp. and *D. immitis* while working dogs living in kennels from Benslimane and Témara were infected more frequently with *A. platys*. In addition to these geographic variations other important factors such as living conditions of dogs influence the prevalence of canine VBPs. Outdoor living has been considered the major risk factor for acquiring a vector-borne infection in dogs[11-13] and several studies showed that rural, stray, hunting, shelter and working dogs are more exposed to these infections.[7,10-12,14-23] In addition, kennel dogs can be more frequently exposed to some TBPs such as *E. canis* and *B. canis*,[6,12] probably due to the particular life cycle of its tick vector tick *R. sanguineus*, also strongly suspected to transmit *A. platys*.[24] Finally, when dogs live in close contact to humans, there is an increased risk of transmission of some zoonotic pathogens. The occurrence of occult infections and coinfections with zoonotic organisms in clinically healthy dogs and humans might result in a complex disease expression in sick dogs and humans.[25-27]

In addition to the occurrence of single exposure to several VBPs, our study demonstrates the existence of frequent co-exposure (16.6%) among the canine populations studied. Dogs can be simultaneously or sequentially infected with a large number of VBPs depending on the presence and abundance of arthropod vectors.[28] Co-infection by multiple VBPs appears to be more frequent in dogs living in endemic areas and particularly in environments in which the vector population density is high.[28-30] Exposure to multiple VBPs has been detected in several serological surveys worldwide with prevalence rates of co-exposure up to 61%.[5,7,12,31-35] In the Mediterranean region including North Africa,

other VBPs than those detected in our study have been reported to infect dogs including *Leishmania* spp., *Bartonella* spp., *Rickettsia* spp., *Babesia* spp. and *Hepatozoon canis*. Co-infection with these VBPs and *Anaplasma* spp., *Ehrichia* spp. or *D. immitis* have also been described.[14,15,32,33,36-40] Considering that co-infections complicate the pathophysiology, clinical manifestations, outcome, diagnosis and management of VBDs,[41,42] the identification of the pathogens circulating in a canine population within a specific area is necessary. In addition, specific diagnostic tools are necessary to help veterinarians in the achievement of an accurate diagnosis and to adapt the management of these diseases. Furthermore, long-term antibody persistence of some select VBPs detected in our study (*Anaplasama* spp., *Ehrlichia* spp.) contributes to the challenges of co-exposure interpretation.[33,35] Moreover, the complex interactions occurring between the host immune system and single pathogens can be modified by simultaneous or sequential infections with multiple pathogens, which may influence the serological and parasitological diagnosis. Indeed, experimental infection with *A. platys* and *E. canis* can alter the anticipated serological response in dogs that were co-infected or sequentially infected compared to that of those infected with a single organism.[43] In light of the detrimental clinical impact associated with VBD co-infections, further characterization of co-exposure epidemiology would benefit both animals and humans.[35]

The frequency of exposure to single and multiple VBPs in Moroccan dogs highlight the importance of adapted diagnostic tools to identify the organism(s) involved in these infections. In Morocco, no laboratory is currently interested in the preparation of diagnostic tests for these pathogens and serological and molecular diagnostic tools for *Anaplasma* spp., *Ehrlichia* spp. and *D. immitis* are lacking. The only available diagnostic tools in some veterinary practices are the cytological diagnosis based on blood smear evaluation and in-clinic serological tests. Consequently, empirical treatments are frequently given to dogs clinically suspected to have *Anaplasma* spp. or *Ehrlichia* spp. infections in Morocco, without considering the possibility of co-infections with other VBPs such as *D. immitis*. Noteworthy, the most frequent co-exposure detected in our study is between *Ehrlichia* spp. and *D. immtis* (7.8%). It has been suggested that the high tick infestation and the subsequent high prevalence of *E. canis*,[44] prompt local veterinary practitioners the indiscriminate use of tetracycline to treat the suspected *Ehrlichia* infection. However, tetracycline therapy may contribute to the reduction of adult worms and its reproduction capability by destroying a filarial endosymbiont *Wolbachia* spp., which could lead to false negative serological results using the SNAP 4DX Plus.[45,46] The diagnosis of canine VBDs is challenging for veterinarians because clinical signs induced by various VBPs may be similar, asymptomatic or subclinical infections are frequent and because co-infections may lead to overlapping or atypical clinical signs.[25-27,47,48] Moreover, direct visualization of *A. platys*, *A. phagocytophilum*, and *E. canis* on blood smears examination of acutely infected dogs might be time-consuming, technically

challenging, and diagnostically insensitive due to the low-level and transient parasitemia[27] and its sensitivity is lower than molecular-based diagnostic tools.[33,49] Serological tests can be very easy to perform due to the development of in-clinic devices. However, they have two main limitations, i.e, the cross-reactions between closely related bacteria such as *Anaplasma* species and *Ehrlichia* species, and the inability to discriminate active infection from prior exposure especially in endemic areas.[27,33,50,51] For *D. immitis*, blood sample examination for the presence of microfilariae and identification based on morphology is considered definitive proof of infection. However, this method is based on the training of the persons examining the blood smear to differentiate *D. immitis* microfilariae from other species (*Dirofilaria repens*, *Acanthocheilonema* spp.). Also, up to 30% of infected dogs do not have circulating microfilariae even though they harbor adult worm. Therefore, the sensitivity of testing for microfilariae is not sufficient to rule out infection in the case of a negative result.[52] The in-clinic tests designed to detect adult heartworm antigens based on ELISA and immunochromatography/lateral flow staining techniques are considered highly specific, but since these tests allow for detection of antigens produced only by female worms, false negative results may occur in infections of less than 5 months duration or very light infections or when only male worms are present.[52] Consequently, the diagnostic confirmation of canine VBDs should take into consideration the historical exposure to arthropod vectors, compatible clinical signs and physical examination findings, biochemistry and hematological abnormalities, and the combination of multiple diagnostic modalities including cytological, serological, and molecular tests.[27,33,41,52] Considering the very limited diagnostic tools available in Morocco, the risk of misdiagnosis might be increased and probably leads to an overuse of tetracycline in dogs clinically suspected to have a VBD, which can impact the serological diagnosis of *D. immitis* infection in dogs. Therefore, negative serological results to *D. immitis* should be interpreted with caution and possible underestimation of the true prevalence of *D. immitis* can occur.

2. Prevalence of *Anaplasma* spp. in dogs in Morocco

Although seroprevalence of *Anaplasma* spp. antibodies in dogs was high (21.9%), only 7.5% of dogs tested positive to *A. platys* by PCR and none tested positive to *A. phagocytophilum*. The discrepancy between the high seroprevalence and the moderate *A. platys* DNA detection could be due to several factors including the cyclic bacteremia associated with this bacterium or DNA degradation in some samples due to transport conditions. Indeed, in experimental *A. platys* infections, morulae appear 8-17 days after inoculation with maximal parasitemia occurring during the initial parasitemic episode about 4 days after the first appearance of morulae and then becoming cyclic at approximately 10-14 day intervals.[53-56] The percentage of platelets containing morulae decreases to as low as 1% or less with subsequent parasitemic episodes, making detection of morulae more difficult.[53,57] Finally, the cyclic nature of the parasitemias diminishes with time, resulting in mild, slowly resolving thrombocytopenia in association with sporadically occurring organisms in blood platelets. Therefore, although appropriately performed PCR-based assays are the most sensitive assays available to diagnose *A. platys* infection, false-negative test results can occur even in acute infections due to the evolution of *A. platys* bacteremia.[24,58] It is hence possible that some dogs sampled in our study had *A. platys* infection below the detection limits of the method used or were in a phase without bacteremia. Another explanation is a resolved infection with only persisting antibodies as a result of previous exposures.

The discrepancy between the high seroprevalence and the moderate *A. platys* infection could also be explained by the circulation of other *Anaplasma* spp. such as *A. phagocytophilum* in the sampled dogs which has not been detected by PCR but could be responsible of an immunological response. This hypothesis could be supported by the fact that other studies form the Mediterranean area found very high prevalence rates up to 57.7% of *A. platys* DNA in dogs.[39] *Anaplasma platys* was thought to be the main *Anaplasma* species in some regions such as South America and Africa. However, studies detected *A. phagocytophilum* DNA in Mediterranean, African and South American countries.[5,17,59-67] Moreover, the geographic distributions of both bacteria can overlap in some regions of the world since a few reports evaluated the occurrence of both bacteria in the same country and detected their DNA.[5,59,63-65] The PCR method used in our study based on the detection of *A. phagocytophilum msp2* gene using a real-time quantitative PCR (TaqMan-PCR) is reported to be highly specific and sensitive[68] and succeeded in detecting *A. phagocytophilum* DNA in regions where other protocols (mainly based on conventional PCR targeting the *16S rRNA* gene) failed to detect it.[61,62,69-70] The use of the *msp2* gene target improves the specificity because it is not present in some more distantly related bacteria such as *Ehrlichia* spp. or *Bartonella spp*., and due to a lower risk of contamination.[69,71,72] The greater analytical sensitivity of the *msp2* assay occurred because of TaqMan's more efficient amplification chemistry.

In addition, this method was designed to amplify the *msp2* gene over a wide variety of *A. phagocytophilum* strains from varying locations.[68] However, although the PCR protocol used in our study is reported to be highly sensitive, false-negative results are reported to occur with molecular-based diagnosis of *A. phagocytophilum* due to the low template concentrations,[50,73] the short duration of bacteremia in dogs and the variation in levels of circulating bacteria.[50,74] Therefore, even when using assays with well-documented sensitivity, clinical specimens from known positive dogs may test negative, particularly when collected at a single time point.[50] Moreover, several studies demonstrated a higher prevalence of *A. phagocytophilum* DNA in clinically ill dogs than in apparently healthy ones,[30,59,75-77] suggesting an association between positive PCR results and clinical illness.[74] Since DNA-based diagnostic tool enables the early detection of the infection by *A. phagocytophilum*, the bacteremia is of short duration and is usually present transiently during the acute phase of the infection,[74,78,79] negative PCR results might be more difficult to interpret in healthy dogs which is probably the case in our study. On the other hand, selective amplification of the predominant organism can occur in patients co-infected with genetically similar organisms[73,80] such as *A. phagocytophilum* and *A. platys*, which could be the case in our study. A recent study on Anaplasmataceae experimental infection in dogs demonstrated that under the same experimental conditions and using the same PCR protocol, *A. platys* was more frequently detected on blood by PCR (92%) than was *A. phagocytophilum* (50%).[56] Therefore, negative PCR results only indicate that the respective nucleic acid sequence was not detected in the sample evaluated under the assay conditions used and should not be interpreted as evidence of absence of infection.[50] Other factors could also explain the negative *A. phagocytophilum*-PCR result mainly the likely degradation of the DNA due to the transport conditions from Morocco to the USA, the circulation of *A. phagocytophilum* strains not detected by the protocol used and the selected region of sampling. The dogs included in our study were sampled exclusively from the western part of Morocco but previous studies detected *I. ricinu* ticks, the main vector of both *A. phagocytophilum* and *B. burdgorferi* in Europe,[81] in the eastern regions of Morocco.[2-4] In addition, *Borrelia burgdorferi*, that is transmitted by *Ixodes* spp. ticks, was reported in dogs in Algeria[7] and ticks in Northeastern Morocco,[2] suggesting that these ticks could be more prevalent in eastern regions of the country.

Although a higher prevalence of *A. platys* infection was recorded in working dogs living in kennels, no risk factor associated with this infection in dogs was identified. Other studies on *A. platys* infection in dogs demonstrated an association with ticks infestation, especially with *R. sanguineus*.[36,48,82] It has been suggested that the prevalence of *A. platys* could be influenced by the structure of the tick community and especially by the abundance of *R. sanguineus* probably because of the short duration of the bacteremia.[43] In a recent study, low prevalence of *R. sanguineus* was associated with the detection of *A. platys* in other *Rhipicephalus* spp. in Kenyan islands.[82] We also did not found any significant

difference between dogs with clinical signs compatible with TBDs and those apparently healthy. In another study, *A. platys*-positive dogs were mostly apparently healthy and displayed only occasional laboratory abnormalities.[83] It has been suggested that more pathogenic strains than those found in the USA are circulating in the Mediterranean region, responsible of more severe clinical signs including lethargy, fever, anorexia, lymphadenomegaly, splenomegaly, abdominal pain, bleeding disorders, purulent nasal discharges more frequent mortality and possible decreased response to doxycycline therapy. Co-infections with other VBPs or intrinsic factors specific to each dogs (age, physical condition, immune status, or stress) may contribute to a more severe expression of the disease.[40,84-93] However, a recent study on naturally infected dogs in the Mediterranean region did not find any significant differences regarding the clinical expression of *A. platys* between mono- and co-infected dogs suggesting that strains virulence and/or other factors (concurrent diseases, genetic factors, immune status, physical condition, stress) could be involved in the more sever clinical expression. Putative immune-mediated processes such as immune-mediated hemolytic anemia or thrombocytopenia might explain the severity of some cases, especially the dogs that died or did not improve despite appropriate medication.[40] Therefore, ICCT should be considered in the differential diagnosis of dogs displaying clinical signs compatible with those aforementioned in Morocco, and co-infections as well as immune-mediated processes should be suspected in treatment cases.

3. *Rhipicephalus sanguineus* ticks and their epidemiological significance

Rhipicephalus sanguineus, commonly called the brown dog tick or kennel tick, is probably the most widely distributed tick species. It is also the most common tick found in the Mediterranean region.[94,95] This tick has been introduced from the Afrotropical Region to many countries in the world, probably by the importation of infested domestic dogs, its preferred host.[96,97] For dogs, the brown dog tick can produce debilitating effects due to both blood loss and the transmission of infectious agents.[96] More recently, a report described suspected paralysis associated with these tick bites in dogs.[98] *Rhipicephalus sanguineus* is known as a competent vector of several pathogens including *Rickettsia* spp., *Babesia canis, Hepatozoon canis* and *E. canis*, is suspected to transmit *Bartonella* spp., *Mycoplasma haemocanis* and *A. platys*[42,98] and could be implicated in the epidemiology of canine visceral leishmaniasis.[99-102] In addition, several other TBPs known to be transmitted by *Ixodes* spp. ticks where detected in *R. sanguineus* including *A. phagocytophilum* and *Borrelia* spp.[62,95,103-105] Several *Rhipicephalus* species including *R. sanguineus* were infected by *A. phagocytophilum* and were suggested as potential competent vectors of this bacterium in the Mediterranean area.[106-111] A report from Jordan detected high prevalence of *A. phagocytophilum* infection in stray dogs and *R. sanguineus* was the most abundant species parasitizing these dogs (95.1%) whereas no *Ixodes* spp. were detected.[17]

Although *A. phagocytophilum* DNA is increasingly reported from *R. sanguineus* ticks, it remains to establish if they play a role in the transmission of this pathogen to humans and other animals.[104] Morphological and molecular data indicated that *R. sanguineus* represents a complex of species and at least four different taxa have been identified under the name *R. sanguineus.*[112-117] Therefore, the potential genetic diversity among *R. sanguineus* species could also influence its vectorial competence in some geographic regions, but more studies are needed to elucidate this issue.[116]

In tropical and subtropical regions, *R. sanguineus* ticks have the particularity to be active throughout the year and to achieve two or more generations per year. Warm temperature may contribute to increased tick abundance by a more rapid development.[118,119] *R. sanguineus* is a nidiculous tick that can complete its entire life cycle either indoor (in houses, kennels and veterinary hospitals where it readily colonizes the infrastructure) or in outdoor environments (peri-urban and rural).[6,96,120,121] *Rhipicephalus sanguineus* populations can reach very high numbers in sheltered environments, because the blood supply necessary for their development is guaranteed by the presence of hosts in close proximity. In dogs without appropriate protection, parasitic loads can reach hundreds of ticks per animal, with ticks in all developmental stages.[122] Although *R. sanguineus* ticks usually feed on dogs, they can also feed in a wide variety of animals including humans.[94-96,121] Due to the close relationship between dogs and humans, some ectoparasites of domestic dogs may parasitize people. This parasitism, though unusual, might be responsible for a simple skin lesion or for the transmission of infectious agents.[96,123] However, several reports described human parasitism with *R. sanguineus* ticks, suggesting that it is more unfrequently reported than unusual and human infestation might be associated with a high level of environmental infestation.[95,96,123-135] Surveys investigating tick infestation in human from southern Europe and southern America found that *R. sanguineus* was amongst the most frequently retrieved tick species,[95,123,133,134] especially in urban areas.[134] One report describing four cases of human parasitism with *R. sanguineus* found several ticks in the house of the patients mainly on the sofa and the wall.[96] Therefore, people living or in daily contact with highly parasitized dogs might be included in the group at risk for parasitism by *R. sanguineus*. Veterinarians and veterinary employee are also included in this group, because of close contact with infested dogs.[96,118] TBDs are recognized as an emerging public health problem in many countries and *R. sanguineus* has been linked to some of these diseases, such as boutonneuse fever caused by *Rickettsia conorii*.[94,96,136] In Europe and the USA, most cases of boutonneuse fever are registered during summer, when *R. sanguineus* ticks are highly active.[136] Similarly, human parasitism by *R. sanguineus* ticks in the USA has been reported to occur predominantly during the summer and fall[126,127] In Morocco, cases of *R. conorii* infection have been reported.[137-139] It has been found that the human affinity of *R. sanguineus* was increased in warmer temperatures, and that there is a warming-mediated increase in the aggressiveness of *R. sanguineus*,

leading to increased human attacks, and more pathogens transmitted by the brown dog tick may emerge in the future as a result of globalization and global warming.[140] This could explain the report of *E. canis* and *A. platys* human infections,[73,120,141-145] and emphasizes the importance of *R. sanguineus*. Therefore, due to its high degree of adaptability, *R. sanguineus* represents a major threat not only to dogs, but also to humans. Consequently, as *R. sanguineus* infestation in dogs was frequent in our study, further investigation should be carried out to better understand the ecology and biology of *R. sanguineus* in Morocco and to assess its public health importance and its ability in transmitting TBPs.

4. Prevalence of *Anaplasma phagocytophilum* exposure in healthy humans in Morocco

This survey is the first to report human exposure to *A. phagocytophilum* in Morocco and in Africa more generally. Our study detected relatively high prevalence rates in both the high risk population composed by military and gendarmerie dog handlers and in blood donors with 27.5% and 21.7% of seropositivity, respectively at the dilution recommended by the WHO (1:128).[146] Our results are quite similar to those found in endemic areas of the USA and Europe.[147-149] In addition, 6 out of 10 dog owners were also seropositive to *A. phagocytophilum* IgG at the same dilution. In Morocco, sparse published surveys are available on TBPs mainly on ticks and domestic animals.[2-4,150-157] Although the tick population is abundant in Morocco and several TBPs of medical importance have been detected, only very few studies on human cases or human exposure are currently available. Meskini et al., reported in 1995 the prevalence of *Rickettsi conorii*, *R. typhy* and *Coxiella burnetti* in two cities of Morocco with rates of 5.6% to 7%, 1.7% to 4% and 1% to 18.3%, respectively; but they failed to detect *Ehrlichia chaffeensis*.[158] Another survey found that 20.5% of patient displaying fever of unknown etiology in northwestern Morocco had tick-borne relapsing fever caused by *Borrelia hispanica* confirmed by PCR.[159] Cases of human rickettsiosis caused by *R. conorii* or *R. aeschlimannii* have also been reported from Moroccan people living in Morocco or in Europe after a stay in Morocco.[137-139,160] The incidence of zoonotic TBDs (anaplasmosis, borreliosis, babesiosis, rickettsiosis) is increasing worldwide. These infections may be associated with both domestic and wild animals with a high risk of acquiring infections for humans frequenting tick-infested areas such as forests, meadow habitats and grassland.[95,161-163] Indeed, the distributions of ticks and thus the risk of pathogen transmission to humans is closely related to the type of environment, often depending on local tick feeding habits and the distribution and density of small-mammal species that act as competent pathogen reservoirs.[28,94,95] Some TBPs such as *A. phagocytophilum*, are more likely related to wild animals as this bacterium is mostly maintained in enzootic cycles involving ticks and wildlife fauna.[164] Dogs and humans are mostly considered as incidental hosts and become infected with *A. phagocytophilum* when they come in contact with the vector in host reservoir habitat.[165] *Ixodes ricinus* the main competent vector of this bacterium in

Europe is an hygrophilous tick species adapted to cool weather that has a high affiliation with wooded areas and pastures.[81,94,133,166] In our study, no *Ixodes* spp. ticks were found infesting dogs in contact with seropositive humans and dogs were exclusively parasitized by *R. sanguineus*. Discrepancies between distributions of *Ixodes* tick species and the pathogens they transmit are reported to occur and are not well understood but may be related to habitat needs, feeding behavior and host-reservoir dynamics.[28] Although dogs were mostly infested by *R. sanguineus* ticks, patients included in this survey could have been previously in contact with other tick species during their outdoor occupational or leisure activities since a high proportion of the blood donors (74.8%) and dog owners (7/10) mentioned having outdoor activities, and dogs handlers are regularly involved in outdoor working. Therefore, the obvious popularity of outdoor activities in the sampled population may have increased the risk of exposure to *A. phagocytophilum*. It has been reported that the kind of activity especially related to outdoor is a conditioning factor for human parasitism by ticks.[123] Indeed, people working or living in rural environments and in forest areas, hunters, national parks rangers and military personnel[167-174] are considered high-risk populations for acquiring *A. phagocytophilum* infection. However, staying indoor is not a warranty of absence of risk for tick infestation.[96,123] A large number of participants to a study from Germany mentioned contracting their most recent tick bite in their gardens and half of the participants with past exposure to *A. phagocytophilum* listed gardening as a regular leisure activity; despite a comparatively low risk of exposure associated with this activity. Additionnally, only a small portion (3.6%) of the tested population had a history of tick exposure. Similarly, several surveys did not find any association between self-reported exposure to ticks and the seroreactivity rates of *A. phagocytophilum phagocytophilum*[175-180] and others demonstrated seropositivity to *A. phagocytophilum* without a history of tick bite.[174] Another report described the highest seropositivity rate to *A. phagocytophilum* among persons who denied having tick bites while the lowest rate was observed in persons who were frequently bitten, probably because the latter are used to checking their body for attached ticks, which may reduce the risk of *A. phagocytophilum* transmission.[178] Several factors can explain this oversight including the stage of feeding ticks and the capacity of ticks to modulate host immune and inflammatory responses, thats may also decrease the chance of detection. Indeed, nymphs and larvae may not be detectable because of their small size.[130,176,177,181] In some studies, nymphal stages of ticks were the predominant stage parasitizing humans, complicating their detection and increasing the risk of pathogens transmission.[95,130,182] The site of attachment on the body can also make the tick detection difficult.[96,181] Furthermore, several persons from the blood donor group that were questioned about previous contact with ticks were not familiar with these parasites and were not able to identify a tick. Ticks can also be confounded with other arthropod parasites such as lice.[123]

Our results showed that even in the blood donor group, high prevalences of 35.7% and 21.7% at the 1:64 and 1:128 dilutions were recorded, respectively. When compared to European prevalences in blood donors using the same method and the same cutoffs, these results are higher than those published in Poland (2%),[177] and Austria (9%),[146] but they are similar to those from Greece (21.4%).[183] Without taking into account the method and the cutoffs, the results from Moroccan blood donors are even higher than those from US and European reports.[167,168,175,184-189] In several reports that compared the seroreactivity rates of blood donors to those of high-risk populations, significant differences were found;[175,186,187] these findings are in contrast to our report. Therefore, our report highlights the potential importance of *A. phagocytophilum* infection in blood donors in Morocco. This infection can be subclinical or asymptomatic especially in endemic areas,[190,191] increasing the risk of sampling infected blood donors. Indeed, it has been suggested that people at high risk for a tick bite have a higher proportion of asymptomatic anaplasmosis.[192] In addition, *A. phagocytophilum* is able to survive in refrigerated blood products up to 18 days.[193] Since this bacterium infects neutrophils, leukoreduction was thought to be able to avoid the risk of transmission through blood transfusion.[194] However, this method did not successfully prevent the transmission of this bacterium in several cases suggesting that it is not efficient in eliminating the risk.[191,195-198] Although transfusion-transmitted *A. phagocytophilum* infection seems to be rare, it is likely to be more severe than the infection acquired after a tick bite[196] probably due to the immune status of these patients and to the administration of immunosuppressive therapy.[190,191] Considering the discrepancy between the seroprevalence and the reported cases in endemic areas, it has been hypothesized that transfusion-transmitted *A. phagocytophilum* infection might be unrecognized in the majority of cases owing to the low bacterial virulence that can be enhanced by immunosuppressive therapy.[190,191,195] Because of the rarity of transfusion-associated cases reported even in endemic areas of the USA, concerns regarding the specificity of available tests, and the economic costs associated with implementation, the blood supply in the USA is not routinely screened for tick-borne disease using laboratory methods.[190] Indeed, in endemic areas of the USA where seroprevalence is high, the chance of accepting a donor with subclinical infection is a less hazardous alternative than using serologic screening with a resultant dramatic reduction in blood supply. In addition, PCR testing of donors would be cost prohibitive and likely low yield. Deferring potential donors in disease-endemic areas during peak tick activity (April-September) would severely limit the blood supply with little potential gain.[195] Furthermore, tick bite–specific screening questions have not proved useful, as donors usually do not remember a tick bite. Donors who do find a tick typically do so within the first 24 to 48 hours, before infective transmission is likely to have taken place; excluding these donors may limit donation by up to 9% in the USA endemic regions.[190,199,200] Similarly, the report of outdoor activities in wooded habitat in an anaplasmosis endemic area is poorly predictive for possible infection.[190] Therefore, in the absence of effective screening tools to identify infected donors or products

and since the incidence of anaplasmosis increases, physicians should suspect *A. phagocytophilum* infection when febrile illness associated with leukopenia or thrombocytopenia develops in a patient after transfusion. Such signs should lead to rapid assessment for rickettsial agents especially *A. phagocytophilum* and empiric treatment with doxycycline.[196,199]

5. Future perspectives

This study demonstrates that Moroccan dogs are frequently exposed to *Anaplasma* spp., *Ehrlichia* spp. and *D. immtis* and detected co-exposures. It has also shown that rural dogs with outdoor living and working dogs living in kennels are more exposed to *Ehrlichia* spp. and *D. immitis*, and infected by *A. platys*, respectively. In addition to the importance of adapted diagnostic tools lacking in Morocco, our study highlights the need for adapted ectoparasites and heartworm preventive programs in all dogs and especially in those with frequent access to outdoor or living in kennels. Several reports demonstrated that ectoparasites prevention is a protective factor against tick-borne infections such as *E. canis* and *A. phagocytophilum*[6,12,14,37,201] or that tick infestation is a risk factor associated with higher prevalence rates.[10,49,62,82,202] Therefore, veterinarians should pay attention to the living conditions of dogs to prescribe the more adapted preventive treatment. Such as the lack of some important diagnostic modalities in Morocco, only a very few ectoparasites preventive treatments are available. Finally, because VBPs can have serious outcome for both canine and human health, dogs can serve as effective sentinels and fluctuations in geographic distribution of vectors and reservoir hosts occur frequently, annual testing of dogs for VBPs exposure and identifying risk factors associated with these infection are crucial.[203] Improved understanding of the geographic distribution, prevalence and risk factors of VBPs and co-exposure in Morocco can facilitate prompt disease diagnosis and effective animal and public health interventions.[34,35,204]

Our study has shown that dogs are infected by *A. platys* but failed to detect *A. phagocytophilum*. The discrepancy between the high seroprevalence to *Anaplasma* spp. and the *A. platys*-positive results by PCR and the lack of specificity of serological tests at the species level could suggest that some of the dogs sampled in this study were exposed to other *Anaplasma* spp. such as *A. phagocytophilum*. Further studies are therefore necessary to evaluate the presence of the later in Morocco. As *I. ricinus*, the most common vector of *A. phagocytophilum* in Europe has been detected in the northeastern of Morocco,[2-4] future surveys must include dogs from this part of the country. Our study was limited to four cities of northwestern Morocco. However, ecological variations between regions of a country can impact the tick populations and thus the associated pathogens that can be transmitted.[9,10] Therefore, large-scale epidemiological surveys are needed to assess the risk for dogs of acquiring *A. phagocytophilum* and

A. platys in each region of the country. Since serological tests cannot discriminate between past exposure and present infection, are unable to identify the *Anaplasma* pathogen at the species level and false negative results can occur with PCR,[50,74,78,79] the combination of both methods is necessary.

The dogs sampled were exclusively parasitized by *R. sanguineus*. This tick species is also known as competent vector of *E. canis* another Anaplasmataceae pathogen widely distributed and responsible for canine monocytic ehrlichiosis. The disease is unspecific and associated with life threatening complications such as glomerulonephritis, meningitis and potential cardiac injury.[204-208] The SNAP 4DX Plus used to detect anti-*Ehrlichia* antibodies does not discriminate between *E. canis*, *E. ewingii* and *E. chaffeensis.*[51] *Ehrlichia chaffeensis* and *E. ewingii* are the causative agents of two tick-borne zoonosis called human monocytis and granulocytic ehrlichiosis, respectively.[209,210] In Africa, these two bacteria are poorly investigated but their DNA has been detected in some tick species including *R. sanguineus* and canine exposure has been reported.[3,211-213] However, these two pathogens and their respective diseases have been described almost exclusively in some regions of the USA where *Amblyomma americanum* is the only proven competent vector.[209,210,212] Therefore, the positivity rates obtained in our study are likely due to the presence of *E. canis* antibodies in the samples tested, especially because this bacterium is prevalent in Africa and the Mediterranean area[5-7,12,19] and that *R. sanguineus* is its main vector. However, since both *E. chaffeensis* and *E. ewingii* are zoonotic and their DNA has been detected in *R. sanguineus* and dogs in Africa, molecular-based epidemiological surveys are needed to clarify which species are circulating in the canine population in Morocco. *Rhipicephalus sanguineus* ticks are competent vectors of other pathogens that can cause serious illness in dogs such as *Babesia canis* and *Hepatozoon canis* and others zoonotic ones such as *R. conorii* and *Bartonella* spp.[33,42,94,98,124,214] In addition, one tick can be infected by and is able to transmit more than one pathogen to a host [28,215] and co-infections are reported to complicate both the diagnosis and the management of the disease.[41,42] Therefore, prevalence data on these infections and co-infections are diagnostically and epidemiologically important for veterinarians and to evaluate the risk of exposure of humans in Morocco, respectively. Future studies should evaluate the occurrence and prevalence of all *R. sanguineus*-transmitted pathogens in dogs in Morocco.

This study is the first to report human exposure to *A. phagocytophilum* in Africa with a high prevalence in dog handlers, owners and blood donors. Although we did not find any risk factor for the seropositivity, blood donors and dog owners frequently reported having outdoor activities. In addition, the occupational activity of dog handlers is frequently associated with outdoor working in divers environments. *Anaplasma phagocytophilum* infection has been frequently associated with outdoor activities especially related to wooded areas.[94,124,162,163] Therefore, studies investigating the occurrence and the prevalence of this bacterium DNA in ticks in parks and forests of Morocco are important to evaluate the risk of exposure and to evaluate the potential vector range. Since our survey detected a high seroprevalence rate in blood donors without difference with the high risk population and higher than the seroprevalence recorded in blood donor in endemic areas of the USA,[195] a large scale study of the prevalence of both exposure and infection with *A. phagocytophilum* in blood donors should be carried in Morocco to better assess the risk of transmission through blood transfusion. In the USA, human granulocytic anaplasmosis in known since several years and is a nationally notifiable disease suggesting that physician are more concerned about this disease.[216,217] In contrast, Moroccan physicians are probably not familiar with this infection and its potential transmission through blood transfusion and hence, adapted screening tools to evaluate the contamination of blood supply are likely to be necessary. In addition, increasing physician awareness to promptly diagnose and treat cases of transfusion-transmitted *A. phagocytophilum* is crucial. This study has been designed to evaluate the occurrence of human exposure in two cities of Morocco and a large-scale survey is needed to evaluate the occurrence and the prevalence of exposure at the national level. In addition, this study was based on the serological screening and thus is only indicative of previous exposure to *A. phagocytophilum*. Future surveys should associate the documentation of seroconversion or a four-fold increase in antibody titer and PCR screening to diagnose an active infection.[149,176] Molecular-based analysis is also important to determine the strain (s) circulating in Morocco since not all are pathogenic for humans.[164,218-221]

The dogs sampled in our study lived in close contact with dog handlers and owners also enrolled. Therefore, due to the close contact and the frequent infestation of these dogs by *R. sanguineus*, dog handlers and owners were at high risk of being infested by this tick species and thus of acquiring a *R. sanguineus*-transmitted pathogen such as *R. conorii.*[96,118,140] Cases of *R. conorii* infection have already been reported in Morocco[137-139] and might be underestimated. In addition although not considered zoonotic, *E. canis* and *A. platys* can also infect human.[73,120,141,144,145] Hence, it is important to determine which pathogens infect *R. sanguineus* ticks in Morocco with a special emphasis on those that are zoonotic or able to infect both dogs and humans. Both the occurrence of *R. sanguineus* infested dogs and *A. phagocytophilum* exposure in humans suggests that people in this study have been exposed to at least two tick species, since *A. phagocytophilum* is mostly transmitted by *Ixodes* spp. ticks.[28,81,205]

Several studies on human parasitism by ticks have shown that the actual diversity of ticks potentially infesting humans is greater than previously believed and any case of human infestation by ticks should be regarded as of clinical significance.[124] Therefore, surveys evaluating human parasitism by ticks and identifying the species and the associated pathogens are warranted. It could also be that *R. sanguineus* plays a role in the transmission of this bacterium in Morocco since the DNA of *A. phagocytophilum* has been detected in this tick species and that some authors suggested that this tick could be a competent vector in the Mediterranean area.[106-111] However, the contact with dogs was not a risk factor *A. phagocytophilum* seropositivity, which is not in favor of this hypothesis. Studies investigating the prevalence of *A. phagocytophilum* DNA using molecular tools in questing ticks and ticks feeding on dogs and humans are mandatory.

Anaplasma platys was known to infect dogs exclusively, but recent reports described human infection.[73,120,144] Since *R. sanguineus* is the most probable competent vector and our dogs were frequently infested by these ticks, we can therefore wonder if the persons included in our study can be infected by this bacterium? Indeed, all human cases of *A. platys* infection reported regular contact with dogs and/or infestation of their dogs with *R. sanguineus*.[73,120,144] In addition, *A. platys* DNA sequencing in two human cases was identical to the sequence found in their dog.[73] All published cases of *A. platys* infection in humans were diagnosed by DNA detection or microscopic identification of morulae within platelets.[73,120,144] In addition, a previous report describing intra-platelet inclusions in humans failed to detect anti-*A. platys* antibodies.[143] Therefore, the occurrence of an immunological response to this bacterium in humans is unknown. Moreover, to our knowledge, the possible occurrence of cross-reactions between *A. platys* and *A. phagocytophilum* antibodies has not been evaluated in humans. The IFA based on HL60-cells infected with a human isolate of *A. phagocytophilum*, such as the one used in our study, are considered to be both sensitive[146] and highly specific for the investigation of seroreactivity to this bacterium[222] with a specificity of 100%, according to the manufacturer. In addition, the absence of relationship between the seropositivity to *A. phagocytophilum* and dogs in our study might be less in favor of an *A. platys* infection. A cross-reaction between antibodies against these two bacteria seems unlikely but we cannot exclude that some of the persons enrolled in our study have had an *A. platys* infection. Consequently, future studies should also investigate the occurrence of *A. platys* infection in humans in Morocco using molecular-based assays.

Our research work implicated the close collaboration between veterinarians, physicians, public health institutions and both humans and veterinary laboratories. Therefore, it is an application of the "One Health" approach. The "One Health" approach as been defined by the American Veterinary Medicine Association as "the collaborative effort of multiple disciplines working locally, nationally and globally to attain the optimal health for people, animals and environment".[223] The "One Health" movement has emerged in the mid of the 20th century due to the increased awareness of zoonotic diseases and embraces a cross-disciplinary, collaborative approach between veterinary and human medicine with clinicians, researchers, agencies and governments working together for the benefit of domestic and wild animal and human health and the global environment to address diseases of importance to both scientific communities. Such interactions may take place at many levels - from management of zoonotic infectious disease outbreaks in the field, to joint research programmes to integrated policy making and funding decisions.[224,225,226,227] It was however, not until the past five years, that the One Health concept has truly gathered international momentum. More recently, the role of companion animals and the VBDs they share with humans have been conceptualized with a One Health approach.[124,224,228]

6. Conclusion of the thesis

In this thesis, we investigated for the first time the exposure of dogs to selected vector-borne pathogens of veterinary and medical significance in Morocco. This first investigation enables us to demonstrate that dogs were frequently exposed to *Anaplasma* spp., *Ehrlichia* spp. and infected by *D. immitis*, with rural dogs at higher risk for *Ehrlichia* spp. and *D. immitis* exposure. This first investigation detected antibodies against *Anaplasma* and *Ehrlichia* genera without discrimination at the species level. Since *A. phagocytophilum* is an emerging zoonotic tick-borne pathogen increasingly recognized worldwide, with potential severe complications, transmitted through blood transfusion and detected in the Mediterranean area and some African countries, we focused on the genus *Anaplasma*.

In the second part of this thesis, we investigated both the exposure to *Anaplasma* spp. and the infections with *A. phagocytophilum* and *A. platys* in a higher number of dogs sampled from three cities of northwestern Morocco. We confirmed that dogs are frequently seropositive to *Anaplasma* spp. without difference according to the living conditions. This study also demonstrates that dogs are infected by *A. platys* but failed to detect *A. phagocytophilum* DNA by PCR. In addition, the only tick species detected on these dogs was *R. sanguineus*, which is considered the most probable vector of *A. platys*. Although *A. phagocytophilum* DNA was not detected, this study cannot exclude the circulation of this bacterium in canine population and further investigations are warranted. In addition, dogs were sampled exclusively in the northwestern part of Morocco but *I. ricinus* ticks have been reported to occur in the northeastern regions. Future surveys should include dogs from the eastern part of the country.

Considering the zoonotic aspect of *A. phagocytophilum* we also evaluated the human exposure to this bacterium in Morocco in the final part of this study. This investigation demonstrated a high seroprevalence in both the high-risk group of dog handlers and the blood donor group without significant difference between both groups. In addition, dog owners were also frequently exposed to this bacterium. Although this study failed to identify risk factors for human exposure to *A. phagocytophilum* in Morocco, a high proportion of the persons sampled reported regular outdoor occupational or leisure activities, which could have increased the risk of exposure to ticks. The seroprevalence rates obtained were similar than those from endemic areas of the USA and Europe. This study is the first to demonstrate human exposure to *A. phagocytophilum* in Africa and highlight its importance in Morocco due to its high seroprevalence.

This thesis investigated canine and human exposure to vector-borne pathogens focusing on *A. phagocytophilum* and *A. platys*. Although it has failed to detect the DNA of *A. phagocytophilum* in dogs, the discrepancy between the high seroprevalence to *Anaplasma* spp. antibodies and the moderate prevalence of *A. platys* DNA in those dogs suggests the possible exposure to other *Anaplasma* species. In addition, the high seroprevalence in humans supports the likely circulation of this bacterium in Morocco and should encourage investigation to better understand the epidemiology of this bacterium and its medical significance.

Above all, this thesis highlights the importance of tick-borne infections in Morocco and the need for further surveys to identify the pathogens circulating in this country, and their veterinary and public health significance.

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SUMMARY

Summary

Zoonotic vector-borne diseases (VBDs) are of increasing interest because they constitute an important emerging threat to both canine and human health. Dogs can play an important epidemiological role in some zoonotic vector-borne pathogens (VBPs) as competent reservoir hosts, carriers of infected vectors in close contact to humans or effective sentinels to assess the risk for human infection. Due to the complexity of VBDs diagnosis and control, as well as the possibility of subclinical infection in dogs that increases the risk of disease transmission, epidemiological data aiming at improving knowledge within a region is fundamental. *Anaplasma phagocytophilum*, *A. platys*, *Borrelia burgdorferi*, *Ehrlichia canis* and *Dirofilaria immitis* are important canine VBPs; some of them are recognized as zoonotic while others are able to infect humans. Among all vectors, ticks are considered to transmit the widest number of pathogens when compared to other arthropod vectors. Some tick-borne pathogens (TBPs) are considered to be emerging because of several factors that play a crucial role in ticks multiplication and expansion, increasing the likelihood of humans and animal tick biting and pathogens transmission. Among these emerging TBPs of zoonotic relevance, *Anaplasma phagocytophilum* is responsible of a widespread disease called granulocytic anaplasmosis. In the past decades, both human and animal exposure has continuously increased in the USA, Europe and some Asian countries. The disease in humans is potentially fatal with severe complications, high hospitalization rates and difficult diagnosis. *Anaplasma phagocytophilum* has been detected in Africa and the Mediterranean region. In these regions, *A. platys* is another *Anaplasma* species causing disease in dogs and able to infect humans. In Africa, only a few studies on *A. phagocytophilum*, *A. platys*, *B. burgdorferi*, *E. canis* and *D. immitis* exposure and/or infection in dogs are available and data on these infections is lacking in Morocco. Similarly, only very few studies on tick-borne diseases (TBDs) in humans in Morocco have been published and no data are currently available on human exposure to *A. phagocytophilum*.

Chapter I explains the importance of VBDs worldwide and emphasizes on the factors that contribute to their expansion and increasing interest. This chapter focusses on TBDs and especially on *A. phagocytophilum*. This section also summarizes the most important epidemiological features of *A. phagocytophilum* and *A. platys* including transmission modes, host reservoir range, life cycle, genetic diversity, zoonotic potential, worldwide distribution and discusses the epidemiological roles of dogs. We conclude that due to the worldwide distribution of *A. phagocytophilum* and *A. platys*, these two bacteria might be present in the canine population in Morocco and humans could be exposed to *A. phagocytophilum*. This led us to the objective of this thesis stated in **Chapter II**: the evaluation of the occurrence of *A. phagocytophilum* in both dogs and humans and *A. platys* in dogs in Morocco.

At the start of this thesis, no data on canine exposure to *Anaplasma* spp., *Ehrlichia* spp. and *Borrrelia burgdorferi* in Morocco were available and only one published study reported *Dirofilaria immitis* infection in a small number of dogs. In **Chapter III**, we investigated the exposure to the four aforementioned VBPs in 217 dogs from seven Moroccan locations using a commercial in-clinic ELISA test. Of these dogs, 83.9% were positive for at least one pathogen and co-exposures were detected in up to 14.3% of the dogs. None of the dog tested seropositive to *B. burgdorferi.* In contrast, antibodies against *Anaplasma spp.* and *Ehrlichia* spp. and *D. immitis* antigens were frequently detected. Statistically significant differences in seropositivity rates were found for *Ehrlichia* spp. and *D. immitis* in rural dogs but not for *Anaplasma* spp. This first part of the thesis demonstrated that Moroccan dogs are at high risk of acquiring a vector-borne infection and detected *Anaplasma* spp. antibodies in the dogs sampled. Since the ELISA test used is not able to discriminate between *A. phagocytophilum* and *A. platys*, we decided to assess the canine infection with these two bacteria.

In **Chapter IV**, we investigated the exposure to *Anaplasma* spp. and infection with *A. phagocytophilum* and *A. platys* in a higher number of dogs (n = 425) from three cities of northwestern Morocco. Canine blood samples were screened for *Anaplasma* spp. antibodies by enzyme-linked immunosorbent assay (ELISA) and for *A. phagocytophilum* and *A. platys* DNA by a quantitative real-time polymerase chain reaction (RT-PCR) targeting the *msp2* gene. The results confirmed that *Anaplasma* spp. antibodies were frequently detected in dogs. The DNA of *A. platys* was also amplified while no dog tested positive to *A. phagocytophilum* by PCR. Although the PCR protocol used is highly sensitive, false-negative results are reported to occur with *A. phagocytophilum* PCR mainly due to the short duration of bacteremia and the variation in levels of circulating bacteria. Therefore, the negative *A. phagocytophilum*-PCR results only indicate that the respective nucleic acid sequence was not detected in the sample evaluated under the assay conditions used in our study and should not be interpreted as evidence of absence of infection in dogs in Morocco. Moreover, the discrepancy between the high seroprevalence to *Anaplasma* spp. antibodies and the moderate prevalence of *A. platys* DNA could suggest that the dogs sampled were potentially exposed to other *Anaplasma* species. Noteworthy, we collected ticks from some of the dogs included in this study. All ticks were identified as *Rhipicephalus sanguineus*, the most probable vector of *A. platys*. Unfortunately, screening of these ticks for *A. phagocytophilum* and *A. platys* DNA was not possible due to the degradation of the DNA.

Currently, no data are available on the occurrence of human exposure to *A. phagocytophilum* in Africa. In **Chapter V**, we evaluated the seropositivity to this bacterium in 271 healthy dog handlers, owners and blood donors from two cities of northwestern Morocco. Indirect immunofluorescent assay using a commercial kit was performed to detect specific *A. phagocytophilum* immunoglobulin G. Two dilutions were used to assess the prevalence of seroreactive samples. Seropositivity rates reached 37% and 27% in dog handlers and 36% and 22% in blood donors, without significant difference between both groups. In addition, 7 and 6 out of 10 owners were also seropositive at the first and second dilutions, respectively. No risk factor was identified but a high proportion of blood donors and dog owners reported regular outdoor activities and dog handlers were frequently involved in outdoor occupational activities. This investigation demonstrates that *A. phagocytophilum* exposure is common in both the high-risk group of dog handlers and blood donors in Morocco, and therefore emphasizes its public health importance.

This study provides important knowledge on canine exposure to *Anaplasma* spp. and *Ehrlichia* spp., and on infection with *A. platys* and *D. immitis* in Morocco. In addition, it provides the first demonstration of human exposure to *A. phagocytophilum* in Morocco and Africa more generally. Our results showed that both dogs and humans in Morocco are frequently exposed to TBPs and emphasize the public health importance of these agents. Our study was designed to evaluate the occurrence of *A. phagocytophilum* and *A. platys* in both dogs and humans in limited regions of the country. Large scale surveys are mandatory to evaluate the risk of exposure in all Moroccan regions. Future studies should evaluate the epidemiological aspects of *A. phagocytophilum* infection (i.e., vectors, reservoir hosts, genetic variability), the risk factors associated with this infection, the public health importance of transfusion-transmitted anaplasmosis and the ability of this bacterium in causing diseases in both dogs and humans in Morocco.

SAMENVATTING

Er bestaat een toenemende belangstelling voor zoönotische vector overdraagbare ziekten omdat ze een belangrijke opkomende bedreiging vormen voor de gezondheid van zowel honden als mensen. Honden kunnen een belangrijke epidemiologische rol spelen bij sommige zoönotische vector overdraagbare ziekten als competente reservoir gastheren, dragers van geïnfecteerde vectoren dichtbij mensen of als effectieve schildwachten om het risico voor humane infecties in te schatten. Omwille van de complexiteit van de diagnose en controle van vector overdraagbare ziekten, en mede door de mogelijkheid dat subklinische infecties bij honden het risico op overdracht van deze ziekten kan doen verhogen, is het bekomen van epidemiologische data om de kennis binnen een streek te verhogen van fundamenteel belang. *Anaplasma phagocytophilum*, *A. platys*, *Borrelia burgdorferi*, *Ehrlichia canis* en *Dirofilaria immitis* zijn belangrijke vector overdraagbare pathogenen bij de hond; sommige van deze pathogenen zijn erkend als zoönosen, terwijl andere de mogelijkheid hebben om mensen te infecteren. Vergeleken met andere geleedpotige vectoren, kunnen teken de meeste pathogenen overdragen. Sommige teken overdraagbare pathogenen worden als opkomend beschouwd omwille van verschillende factoren die een cruciale rol spelen bij de vermenigvuldiging en uitbreiding van de tekenpopulatie, leidend tot een toename van de kans op overdracht van pathogenen naar mensen en dieren na een tekenbeet. Bij deze opkomende teken overdraagbare pathogenen van zoönotisch belang, is *Anaplasma phagocytophilum* verantwoordelijk voor een wijd verspreide ziekte genaamd granulocytaire anaplasmose. In de laatste decennia is de blootstelling aan deze infectieziekte bij mens en dier toegenomen in de Verenigde Staten van Amerika, Europa en sommige Aziatische landen. De ziekte is bij mensen mogelijk fataal met ernstige complicaties, een hoge graad van hospitalisatie en een moeilijke diagnose. *Anaplasma phagocytophilum* werd reeds gedetecteerd in Afrika en in het Middellandse Zeegebied. In deze regio's vormt *A. platys* een andere *Anaplasma* species die ziekte veroorzaakt bij honden en ook mensen kan infecteren. In Afrika zijn slechts enkele studies beschikbaar die de blootstelling aan en/of de infectie van honden met *A. phagocytophilum*, *A. platys*, *B. burgdorferi*, *E. canis* en *D. immitis* beschrijven. Informatie over deze infecties zijn afwezig in Marokko. Evenzeer zijn er slechts enkele studies over teken overdraagbare ziekten bij mensen in Marokko gepubliceerd, en is er momenteel geen informatie beschikbaar over humane blootstelling aan *A. phagocytophilum*.

Hoofdstuk I handelt over het wereldwijde belang van vector overdraagbare ziekten en legt de nadruk op factoren die bijdragen aan hun uitbreiding en toenemend belang. Dit hoofdstuk spitst zich toe op teken overdraagbare ziekten en meer specifiek vooral op *A. phagocytophilum*. In dit hoofdstuk wordt ook een samenvatting gemaakt van de meest belangrijke epidemiologische kenmerken van *A. phagocytophilum* en *A. platys*, onder andere de wijzen van overdracht, de mogelijke reservoir gastheren, de levenscyclus, de genetische diversiteit, het zoönotische potentieel, de wereldwijde distributie en wordt de epidemiologische rol van honden hierbij besproken. We concluderen dat, door de

wereldwijde distributie van *A. phagocytophilum* en *A. platys*, deze twee bacteriën mogelijk aanwezig zijn in de hondenpopulatie van Marokko, en dat mensen mogelijk blootgesteld worden aan *A. phagocytophilum*. Dit leidde ons tot de doelstelling van deze thesis in **Hoofdstuk II**: de evaluatie van het voorkomen van *A. phagocytophilum* bij honden en mensen en van *A. platys* bij honden in Marokko.

Bij de start van deze thesis was er geen informatie gekend over de blootstelling aan *Anaplasma* spp., *Ehrlichia* spp. en *Borrrelia burgdorferi* bij honden in Marokko, en slechts één gepubliceerde studie beschreef een infectie met *Dirofilaria immitis* bij een kleine groep van honden. In **Hoofdstuk III** onderzoeken we de blootstelling aan de vier hierboven genoemde vector overdraagbare pathogenen bij 217 honden op zeven verschillende locaties in Marokko met behulp van een commercieel beschikbare in-huis ELISA test. Van deze honden waren 83.9% positief voor minstens één pathogeen en werden meerdere blootstellingen tegelijk vastgesteld bij 14.3% van de honden. Geen enkele hond testte seropositief voor *B. burgdorferi*. Antistoffen tegenover *Anaplasma* spp. En *Ehrlichia* spp. en *D. immitis* antigenen werden daarentegen frequent gedetecteerd. Voor *Ehrlichia* spp. en *D. immitis*, maar niet voor *Anaplasma* spp., werden statistisch significante verschillen gevonden wat betreft de mate van seropositiviteit bij honden op het platteland. Dit eerste deel van de thesis toont aan dat Marokkaanse honden een hoog risico hebben om een vector overdraagbare infectie op te lopen, en ook werden antistoffen tegenover *Anaplasma* spp. gedetecteerd bij honden. Aangezien de gebruikte ELISA test geen onderscheid kan maken tussen *A. phagocytophilum* en *A. platys*, besloten we om de infectie met deze twee bacteriën bij honden verder te onderzoeken.

In **Hoofdstuk IV** onderzoeken we de blootstelling aan *Anaplasma* spp. en de infectie met *A. phagocytophilum* en *A. platys* bij een groter aantal honden (n = 425) afkomstig uit drie steden in noordwestelijk Marokko. Bloedstalen van honden werden gescreend voor *Anaplasma* spp. antistoffen door middel van een enzyme-linked immunosorbent assay (ELISA), en voor *A. phagocytophilum* en *A. platys* DNA door middel van een real-time polymerase chain reaction (RT-PCR) van het *msp2* gen. De resultaten bevestigen dat *Anaplasma* spp. antistoffen frequent gevonden worden bij honden. Het DNA van *A. platys* werd ook geamplificeerd, terwijl geen enkele hond positief testte voor *A. phagocytophilum* door middel van PCR. Hoewel het gebruikte PCR protocol zeer sensitief is, wordt beschreven dat vals negatieve resultaten bij *A. phagocytophilum* PCR kunnen optreden voornamelijk ten gevolge van de korte duur van de bacteriëmie en door de variatie in aantal circulerende bacteriën. Daarom tonen de negatieve *A. phagocytophilum*-PCR resultaten enkel aan dat de respectievelijke nucleïnezuursequentie niet gedetecteerd werd in de geëvalueerde stalen onder de omstandigheden van de assay in onze studie, en bijgevolg dat deze resultaten niet geïnterpreteerd mogen worden als bewijs van afwezigheid van infectie bij honden in Marokko. De discrepantie tussen de hoge seroprevalentie van *Anaplasma* spp.

antistoffen en de matige prevalentie van *A. platys* DNA zou kunnen suggereren dat deze honden mogelijk blootgesteld werden aan andere *Anaplasma* species. We verzamelden ook teken van sommige honden die geïncludeerd werden in deze studie. Alle teken werden geïdentificeerd als *Rhipicephalus sanguineus,* de meest waarschijnlijke vector van *A. platys*. Het was helaas niet mogelijk om deze teken te screenen voor *A. phagocytophilum* en *A. platys* DNA omwille van degradatie van het DNA.

Momenteel zijn er geen data beschikbaar over het voorkomen van humane blootstelling aan *A. phagocytophilum* in Afrika. In **Hoofdstuk V** evalueren we de seropositiviteit tegenover deze bacterie bij 271 gezonde hondenverzorgers, hondeneigenaars en bloeddonoren afkomstig uit twee steden in noordwestelijk Marokko. Een indirecte immunofluorescentietest van een commercieel beschikbare kit werd gebruikt om specifiek immunoglobuline G van *A. phagocytophilum* te detecteren. Twee verdunningen werden gebruikt om de prevalentie van seroreactieve stalen te evalueren. Seropositiviteit bereikte 37% en 27% bij hondenverzorgers en 36% en 22% bij bloeddonoren, zonder een significant verschil tussen beide groepen. Daarnaast waren 7 en 6 van de 10 hondeneigenaars ook seropositief bij respectievelijk de eerste en tweede verdunning. Risicofactoren werden niet geïdentificeerd, maar een hoge proportie van de bloeddonoren en hondeneigenaars vermeldden wel regelmatige activiteiten buitenshuis en de hondenverzorgers waren frequent betrokken bij beroepsmatige activiteiten buitenshuis. Dit onderzoek toont aan dat blootstelling aan *A. phagocytophilum* vaak voorkomt in Marokko, zowel bij de hoog risicogroep van de hondenverzorgers als bij de bloeddonoren. Deze resultaten benadrukken bijgevolg het belang van *A. phagocytophilum* voor de volksgezondheid.

Deze studie levert ons belangrijke kennis over de blootstelling aan *Anaplasma* spp. en *Ehrlichia* spp., en over infecties met *A. platys* en *D. immitis* bij honden in Marokko. Daarnaast heeft deze studie voor het eerst de blootstelling aan *A. phagocytophilum* aangetoond bij mensen in Marokko en meer algemeen in Afrika. Onze resultaten toonden aan dat zowel honden als mensen in Marokko frequent worden blootgesteld aan teken overdraagbare pathogenen en benadrukken onze resultaten het belang van deze ziekten voor de volksgezondheid. Onze studie werd opgesteld om het voorkomen van *A. phagocytophilum* en *A. platys* bij honden en mensen in beperkte regio's van het land te evalueren. Grootschalige onderzoeken zijn noodzakelijk om het risico op blootstelling in alle Marokkaanse regio's te evalueren. Toekomstige studies zouden de epidemiologische aspecten van *A. phagocytophilum* infecties (i.e., vectoren, reservoir gastheren, genetische variabiliteit), de risicofactoren geassocieerd met deze infectie, het belang voor de volksgezondheid van anaplasmosis overgedragen door transfusie en de mogelijkheid van deze bacterie om ziekte te veroorzaken bij zowel honden als mensen in Marokko, kunnen onderzoeken.

RESUME

Résumé

Les zoonoses vectorielles présentent un intérêt croissant car elles constituent une menace émergente pour la santé publique et animale. Les chiens peuvent jouer un rôle épidémiologique dans de nombreuses zoonoses vectorielles en tant que réservoirs d'agents pathogènes, transporteurs de vecteurs infectés au contact de l'Homme ou sentinelle dans l'évaluation du risque d'infection pour l'Homme. Etant donné la complexité du diagnostic et du contrôle des maladies vectorielles ainsi que l'existence d'infection asymptomatiques chez le chien augmentant le risque de transmission des pathogènes aux vecteurs, les données épidémiologiques au sein d'une région sont fondamentales. *Anaplasma phagocytophilum*, *A. platys*, *Borrelia burgdorferi*, *Ehrlichia canis* et *Dirofilaria immitis* sont d'importants agents pathogènes à transmission vectorielle reconnus comme zoonotiques pour certains ou ayant la capacité d'infecter l'Homme pour d'autres. Les tiques sont considérées comme les vecteurs transmettant le plus grand nombre d'agents pathogènes en comparaison avec les autres arthropodes vecteurs. Certaines maladies transmises par les tiques sont considérées comme émergentes du fait de la contribution de différents facteurs jouant un rôle crucial dans la multiplication et l'expansion territoriale des tiques et par conséquent, augmentant le risque d'infestation par les tiques et de transmission d'agents pathogènes à l'Homme et à l'animal. Parmi les agents pathogènes transmis par les tiques émergents et zoonotiques, *A. phagocytophilum* est responsable d'une maladie de distribution mondiale nommée « anaplasmose granulocytaire ». Durant les dernières décennies, le nombre d'exposition humaine et animale à *A. phagocytophilum* a continuellement augmenté aux Etats Unis d'Amérique, en Europe et dans certains pays d'Asie. L'infection chez l'Homme est potentiellement mortelle, de diagnostic difficile et peut entraîner de sévères complications associées à des taux d'hospitalisation élevés. *A. phagocytophilum* a été détectée dans des pays d'Afrique du nord et du bassin méditerranéen. Dans ces régions, une autres espèce d'*Anaplasma*, *A. platys*, pathogène pour le chien et capable d'infecter l'Homme est également présente. En Afrique, très peu d'études on été menées sur l'exposition et/ou l'infection canine par *A. phagocytophilum*, *A. platys*, *B. burgdorferi*, *E. canis* and *D. immitis* et ces données sont manquantes au Maroc. De même, très peu d'études sur les maladies transmises par les tiques chez l'Homme sont disponibles au Maroc et aucune donnée concernant l'exposition humaine à *A. phagocytophilum* n'est actuellement publiée.

Le **Chapitre I** explique l'importance des maladies vectorielles dans le monde et met en relief les facteurs contribuant à leur expansion et l'intérêt croissant suscité par ces maladies, en insistant sur celles transmises par les tiques et plus particulièrement sur *A. phagocytophilum*. Ce chapitre résume également les plus importantes caractéristiques épidémiologiques d'*A. phagocytophilum* et d'*A. platys* comprenant les modalités de transmission, les hôtes réservoirs, les cycles de transmissions, la diversité génétique, le potentiel zoonotique, la distribution mondiale et discute le rôle épidémiologique du chien dans ces deux infections. Du fait de la distribution mondiale d'*A. phagocytophilum* et d'*A. platys*, ces deux bactéries

devraient être présentes et circuler au sein de la population canine au Maroc et l'Homme pourrait être exposé à *A. phagocytophilum* étant donné son caractère zoonotique. Par conséquent, l'objectif de ce travail de thèse exposé dans le **Chapitre II** est l'évaluation de la possible circulation d'*A. phagocytophilum* chez le chien et l'Homme, et d'*A. platys* chez le chien au Maroc.

Au commencement de ce travail de thèse, aucune donnée concernant l'exposition canine à *Anaplasma* spp., *Ehrlichia* spp. et *Borrrelia burgdorferi* au Maroc n'était publiée et seul un précédent article a décrit l'infection par *D. immitis* chez un petit nombre de chiens. Dans le **Chapitre III**, l'exposition à ces quatre agents vectoriels a été étudiée chez 217 chiens prélevés dans sept villes marocaines en utilisant un kit ELISA rapide. Parmi les chiens prélevés, 83.9% ont présenté des résultats positifs pour au moins un agent pathogène. L'exposition simultanée à au moins deux agents pathogènes a été observée chez 14 .3% des chiens. Aucun chien séropositif pour *B. burgdorferi* n'a été détecté. A l'inverse, les anticorps anti-*Anplasma* spp. et *Ehrlichia* spp. ainsi que les antigènes de *D. immitis* ont été fréquemment détectés. Une différence significative dans la prévalence à *Ehrlichia* spp. et *D. immitis* a été notée pour le groupe de chiens ruraux mais pas pour *Anaplasma* spp. Cette première partie de notre étude a démontré que les chiens au Maroc présentent un risque important d'infection par des agents vectoriels et a détecté les anticorps anti-*Anaplasma* spp. dans la population de chien prélevés. Etant donné que le test ELISA utilisé ne permet pas de différencier entre une exposition à *A. phagocytophilum* ou à *A. platys*, nous avons décidé d'évaluer la présence de l'infection par ces deux bactéries chez le chien.

Dans le **Chapitre IV**, nous avons étudié simultanément l'exposition à *Anaplasma* spp. et l'infection par *A. phagocytophilum* et *A. platys* dans un effectif canin plus important (n = 425) prélevés dans trois villes du nord-ouest du Maroc. Les anticorps anti-*Anaplasma* spp. ont été recherchés par une méthode immuno-enzymatique ELISA et l'ADN d'*A. phagocytophilum* et d'*A. platys* par une technique de réaction de polymérase en chaine quantitative en temps réel (RT-PCR) ciblant le gène *msp2*. Les résultats obtenus confirment ceux de la précédente étude démontrant encore une fois que les anticorps anti-*Anaplasma* spp. sont fréquemment détectés chez les chiens prélevés. L'ADN d'*A. platys* a également été amplifiée tandis qu'aucun chien n'a été positif à l'ADN d'*A. phagocytophilum*. Malgré la sensibilité du protocole de PCR utilisé, des résultats faussement négatifs peuvent exister principalement dus à la courte durée de la bactériémie et aux variations du nombre de bactéries circulantes. Par conséquent, un résultat négatif lors de la recherche de l'ADN d'*A. phagocytophilum* par PCR signifie uniquement que la l'acide nucléique recherché n'a pas été détecté dans l'échantillon examiné sous les conditions du protocole utilisé et ne devrait en aucun cas être interprété comme une absence de cette bactérie chez le chien au Maroc. De plus, la disproportion entre une forte séroprévalence à

Anaplasma spp. et une prévalence modérée de détection *d'A. platys* pourrait suggérer que les chiens prélevés ont potentiellement été exposés à d'autres espèces du genre *Anaplasma*. Il est par ailleurs important de noter que les tiques prélevées sur ces chiens ont été exclusivement identifiées comme appartenant à l'espèce *Rhipicephalus sanguineus*, le vecteur présumé d'*A. platys*. Malheureusement, la recherche de l'ADN d'*A. phagocytophilum* et d'*A. platys* dans les tiques prélevées n'a pas pu être réalisée à cause de la dégradation de l'ADN.

Actuellement aucune donnée sur la présence de l'exposition humaine à *A. phagocytophilum* en Afrique n'est disponible. Dans les **Chapitres IV et V**, nous avons évalué la séropositivité à cette bactérie chez 271 patients cliniquement sains et subdivisés en trois groupes: les maîtres-chiens, les propriétaires de chiens et les donneurs de sang prélevés dans deux villes du nord-ouest du Maroc. Un kit commercial d'immunofluorescence a été utilisé pour détecter les immunoglobulines G spécifiques à *A. phagocytophilum*. Deux dilutions ont été réalisées pour évaluer la réactivité des échantillons. La proportion de patients séropositifs a été de 37% et 27% chez les maitres-chiens et de 36% et 22% chez les donneurs de sang, sans différence significative entre les deux groupes. De plus, les anticorps dirigés contre *A. phagocytophilum* ont été détectés chez 7 et 6 parmi 10 propriétaires de chiens à la première et deuxième dilution, respectivement. Aucun facteur de risque associé à la séropositivité n'a été identifié. Cependant, un nombre important de donneurs de sang et de propriétaires ont reporté avoir fréquemment des activités en plein air. Cette étude a démontré que l'exposition à *A. phagocytophilum* est fréquente à la fois dans la population à risque et chez les donneurs de sang au Maroc et souligne son importance en terme de santé publique.

Cette étude fournit des données de base sur l'exposition à *Anaplasma* spp., *Ehrlichia* spp. et l'infection par *A. platys* et *D. immitis* au Maroc. Elle fournit également la première démonstration de l'exposition humaine à *A. phagocytophilum* au Maroc et en Afrique plus généralement. Les résultats obtenus ont montré que les chiens et l'Homme sont fréquemment exposés aux agents pathogènes transmis par les tiques et soulignent leur importance en termes de santé publique. Cette étude a été conçue afin d'évaluer la présence d'*A. phagocytophilum* et d'*A. platys* chez le chien et l'Homme dans un nombre limité de villes marocaines. Des études à l'échelle nationale sont nécessaires afin d'évaluer le risque d'exposition à ces bactéries dans toutes les régions du Maroc. Les prochains travaux devraient également étudier les différents aspects épidémiologiques de l'infection à *A. phagocytophilum* (les différentes espèces de tiques potentiellement vectrices, les hôtes réservoirs, la diversité génétique), évaluer les facteurs de risque associés à cette infection, l'importance en terme de santé publique de la transmission de cette infection par transfusion sanguine et la pathogénicité de cette bactérie chez le chien et l'Homme au Maroc.

ملخص

ملخص

تعرف الأمراض الحيوانية المنشأ اهتماما متزايدا لما تشكله من خطر كبير على صحة الحيوانات والعموم، فالكلاب قادرة على لعب دور وبائي في العديد من الأمراض الحيوانية المنشأ كما أنها قادرة أيضا على لعب دورمخزن العوامل المسببة للمرض والناقل للعوامل المصابة والتي بإمكانها إصابة الإنسان سواء بالاتصال المباشر أو بتقديم عدوى للبشر. خطر انتقال مسببات الأمراض لدى الناقلات يزيد نظرا لصعوبة تشخيص وتتبع الأمراض المنقولة عبر الحشرات، فضلا عن وجود عدوى عديمة الأعراض لدى الكلاب، كما أن المعطيات اللازمة والضرورية حول الوباء في المنطقة تعتبر أمرا أساسيا.

هي *A. phagocytophilum, A. platys, Borrelia burgdorferi, Ehrlichia canis* et *Dirofilaria immitis* أهم العوامل المسببة للمرض التي تنتقل عبر ناقلات معلومة كالحيوانية المنشأ لدى البعض أو التي لها القدرة على إصابة الإنسان لدى البعض الآخر. مقارنة بالناقلات المفصلية الأخرى يعد القراد الناقل الحامل لأكبر عدد من مسببات المرض. بعض الأمراض المنتقلة عبر القراد تعتبر ناشئة أو فجائية نتيجة مساهمة العوامل المختلفة والتي تلعب دورا حاسما في نمو وتكاثر القراد وبالتالي زيادة خطر الإصابة عبر القراد وانتقال العوامل المسببة للمرض للإنسان والحيوان. ومن بين العوامل المسببة للمرض المتناقلة عبر القراد الناشئ والحيواني المنشأ نجد *A. phagocytophilum* المسؤول عن التوزيع العالمي للمرض المسمى «anaplasmose granulocytaire ».

خلال العقود الأخيرة ازداد عدد الإصابات لدى الإنسان والحيوان نتيجة *A. phagocytophilum* بشكل مستمر غير منقطع في الولايات المتحدة الأمريكية وأوروبا وبعض الدول الأسيوية.

إن الإصابة لدى الإنسان غالبا ما تكون قاتلة وصعبة التشخيص كما أنها تسبب مضاعفات خطيرة ذات معدلات استشفائية عالية. وقد تم الكشف عن *A. phagocytophilum* في بلدان شمال أفريقيا والبحر الأبيض المتوسط. حيث أن في هذه المناطق نوع أخر من d'*Anaplasma, A. platys* و التي تشكل مسببا للمرض بالنسبة للكلاب و قادرة أيضا على إصابة الإنسان.

في إفريقيا، دراسات قليلة هي التي أجريت عن تعرض أو عدوى الكلاب ب: *A. phagocytophilum, A. platys, B. burgdorferi, E. canis, D. immitis*، علما أن هذه المعطيات مفقودة و منعدمة في المغرب. كما أن الدراسات التي أجريت حول الإمراض المتناقلة عبر القراد لدى الإنسان قليلة جدا بالمغرب فيما المعطيات حول تعرض الإنسان للمرض عبر *A.phagocytophilum* لم تنشر أي منها.

يؤكد الفصل الأول من هذا البحث، أهمية الأمراض المنقولة عبر الحشرات في العالم، كما يسلط الضوء على العوامل المساهمة في انتشارها والاهتمام المتزايد بها، مؤكدا على تلك التي تتناقل عبر القراد خاصة *A. phagocytophilum*. ويلخص هذا الفصل كذلك، الخصائص الوبائية ل *A. phagocytophilum* و *A. platys* إضافة إلى طرق الانتقال، الخزانات المضيفة، دورات الانتقال، التنوع الوراثي، إمكانية الظهور الحيواني، التوزيع العالمي، كما أنه يناقش الدور الوبائي للكلب في كلا الإصابتين، ونتيجة التوزيع العالمي

ل *A. phagocytophilum* و *A. platys* فإن هذه البكتيريا من المؤكد أن تكون حاضرة ومتداولة لدى الكلاب في المغرب، حيث يمكن للإنسان التعرض ل *A. phagocytophilum* ولخصائصه الحيوانية. ولذلك فإن الهدف من هذه الأطروحة المقدم في الفصل الثاني من البحث، هو تقييم إمكانية تدفق محتمل ل *A. phagocytophilum* لدى الإنسان و الحيوان و *A. platys* لدى الكلاب في المغرب.

والجدير بالذكر أنه لم يكن هناك أي بحث في الموضوع في المغرب قبل بداية هذه الأطروحة، كما لم يتم نشر أي معطيات في بلادنا حول تعرض الكلاب ل *Ehrlichia* spp، *Anaplasma* spp، *Borrrelia burgdorferi* فلقد كُتب مقال واحد فقط ، تناول تعرض عدد قليل من الكلاب للإصابة عن طريق *D. immitis*.

في الفصل الثالث من هذا البحث تمت دراسة التعرض نتيجة هذه العوامل الأربع لدى 217 كلبا من سبع مدن مغربية باستعمال عدة ELISA السريعة. 83.9% من عينات الكلاب المدروسة قدمت نتائج إيجابية حول مسبب واحد للمرض على الأقل. كما أن 14.3 % من الكلاب تعرضت في نفس الوقت لمسببين للمرض على الأقل. في حين لم يتم رصد أي كلب يعاني من فيروس نقص المناعة ل *B. burgdorferi* على غرار مضادات الأجسام anti-*Ehrlichia* spp وكذلك مولدات مضادات *D. immitis* التي تم رصدها كثيرا. كما أن هناك فرق هام في الانتشارل *Ehrlichia* spp و *D. immitis* تم تسجيله لدى مجموعة من الكلاب القروية على عكس *Anaplasma* spp.

لقد أظهر الجزء الأول من دراستنا أن الكلاب في المغرب تشكل خطرا بالنسبة للإصابة عبر هذه العوامل الناقلة كما أنها رصدت مضادات الأجسام وanti-*Anaplasma* spp لدى عينات الكلاب المدروسة. و كون اختبار ELISA لم يمكننا من التمييز بين التعرض ل *A. phagocytophilum* أو ل *A. platys* ، قررنا تقييم وجود العدوى عن طريق هذه البكتيريا لدى الكلب.

أما في الفصل الرابع، فلقد قمنا في نفس الوقت، بدراسة التعرض لل*Anaplasma* spp والإصابة ب *A. phagocytophilum* و *A. platys* لدى عينة مهمة من الكلاب تضم 425 فردا، مأخوذة من ثلاث مدن شمال غرب المغرب. كما تم البحث عن مضادات الأجسام anti-*Anaplasma* spp بواسطة طريقة منعاتية-أنزيمية، والحديث هنا عن ELISA والحمض النووي ل *A. phagocytophilum* و *A. platys* عن طريق تقنية تفاعل البلمرة بتسلسل كمي عبر الزمن (RT-PCR) مستهدفة الجينات msp2، حيث تؤكد هذه النتائج نتائج الدراسة السابقة، مبرهنة من جديد على أن مضادات الأجسام anti-*Anaplasma* spp قد تم رصدها كثيرا لدى عينات الكلاب المدروسة. كما تم تضخيم حمض *A. platys* النووي في حين لم يكن أي كلب إيجابيا بخصوص حمض *A. phagocytophilum* النووي.

وعلى الرغم من حساسية بروتوكول PCR، فقد تحدث نتائج سلبية كاذبة، ويرجع ذلك أساسا إلى قصر مدة تجرثم الدم والتغيرات في عدد من البكتيريا المنتشرة. لذلك فإن نتيجة سلبية واحدة عند البحث عن حمض *A. phagocytophilum* النووي بطريقة PCR، تعني فقط أن الحمض النووي المبحوث عنه لم يتم رصده في العينة المفحوصة وفقا لأحكام البروتوكول المعمول به، و لا ينبغي في أي حال من الأحوال، أن تفسر بعدم وجود هذه البكتيريا لدى الكلاب بالمغرب. بالإضافة إلى ذلك، فإن عدم التناسب بين ارتفاع الانتشار المصلي لـ*Anaplasma* spp واعتدال انتشار رصد *A. platys* يمكن أن يشير إلى تعرض الكلاب إلى أنواع أخرى من *Anaplasma* spp المصلي الانتشار. ومن المهم أيضا أن نلاحظ أن القراد المأخوذ من عينات الكلاب المدروسة تم تحديده على انه ينتمي للنوع *Rhipicephalus sanguineus* الناقل المشهور لـ *A. platys*. وللأسف لم يتم تطبيق البحث عن حمض *A. phagocytophilum* و *A. platys* النووي لدى هذا النوع من القراد نظرا لتدهور الحمض النووي.

تجدر الإشارة إلى أن المعطيات حول تعرض الإنسان لـ *A. phagocytophilum* غير متوفرة في إفريقيا حاليا.

وفي الفصلين الرابع والخامس قمنا بتقييم حالة هذه البكتيريا لدى 217 من المرضى السريريين وتم تقسيمهم إلى ثلاث مجموعات: مدربي الكلاب، أصحاب الكلاب والمتبرعون بالدم في مدينتين شمال غرب المغرب. وقد تم استخدام عدة منعاتية تجارية للكشف عن immunoglobulines G الخاصة بـ *A. phagocytophilum*. كما أجريت اثنين من التخفيفات لتقييم تفاعل العينات و بلغت نسبة المرضى المصابين 37 % و 27 % بالنسبة لمدربي الكلاب، و **36** % و 22% بالنسبة للمتبرعين بالدم دون أي فرق مهم بين المجموعتين. إضافة إلى انه تم كشف الأجسام المضادة anti-*A. phagocytophilum* لدى 7 و 6 من عشرة مربيي الكلاب عند التخفيف الأول و الثاني على التوالي. ولم تحدد أي عوامل الخطر المرتبطة بفيروس نقص المناعة البشرية. ومع ذلك، فقد أفاد عدد كبير من المتبرعين بالدم وأصحاب الكلاب أن أنشطتهم غالبا ما تكون في الهواء الطلق. وأظهرت هذه الدراسة أن التعرض لـ*A. phagocytophilum* هوشائع لدى كل من السكان المعرضين للخطر والمتبرعين بالدم في المغرب، مؤكدة أهميته في مجال الصحة العمومية.

تقدم هذه الدراسة معطيات مهمة وأساسية حول التعرض لـ *Anaplasma* spp, *Ehrlichia* spp وكذا الإصابة بـ *A. platys* و *D. immitis* بالمغرب. كما توفر أيضا أول تعليل ودليل حول تعرض الإنسان لـ *A. phagocytophilum* في المغرب وإفريقيا عموما.

وقد أظهرت نتائج هذا البحث، أن الكلاب والإنسان غالبا ما يتعرض لمسببات الأمراض التي تنتقل عن طريق القراد، مؤكدة على أهميتها في مجال الصحة العمومية. كما قد صممت هذه الدراسة لتقييم حضور *A. phagocytophilum* و*d'A. platys* لدى الكلاب والإنسان في عدد محدود من المدن المغربية. إضافة إلى الحاجة الماسة لدراسات على الصعيد الوطني لتقييم مخاطر التعرض لهذه البكتيريا في جميع مناطق المغرب. ويجب العمل في المستقبل على دراسة مختلف الجوانب الوبائية لعدوى

ال *A. phagocytophilum*. (أنواع القراد خاصة الناقلة منها، الخزان المضيف، والتنوع الجيني) وتقييم عوامل الخطر المرتبطة بهذا المرض، بالإضافة إلى تقييم أهمية انتقال المرض عبر نقل الدم ومرضية هذه البكتيريا لدى الإنسان والكلاب في المغرب، وفق سياق سياسة الصحة العمومية.

CURRICULUM VITAE

Sarah El Hamiani Khatat was born on February 15, 1985 in Rabat, Morocco. In 2003, she graduated from secondary education in the direction of Science at the Lycée Descartes in Rabat, Morocco. Immediately afterwards she started her Veterinary Medicine curriculum at l'Institut Agronomique et Vétérinaire Hassan II, Rabat, Morocco. In 2009, she graduated with honors from the Veterinary Medicine degree. After her graduation, she spent two years between 2010 and 2012 at the National Veterinary School of Maisons-Alfort, France where she did an internship in companion animals followed by a specialized internship in internal medicine of companion animals.

The two years spent in the National Veterinary School of Maisons-Alfort gave her the desire to follow an academic carrier. She started a PhD on the field of zoonotic vector-borne diseases and more specifically on *Anaplasma* species. This study was jointly supervised by the Veterinary Faculty of Ghent University and l'Institut Agronomique et Vétérinaire Hassan II of Rabat, Morocco. This work had also the support of the National Reference Laboratory for *Anaplasma phagocytophilum* in Brussels, IDEXX Laboratories in Sacramento, California, USA and the Gendarmerie Services of Rabat, Morocco.

Sarah El Hamiani Khatat has authored or co-authored several scientific publications and presentations.

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