GHENT UNIVERSITY

FACULTY OF VETERINARY MEDICINE

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EPIDEMIOLOGY OF CANINE AND EQUINE PIROPLASMOSIS AND THEIR VECTOR IN EUROPE

by

Michael MEIJER

Promoters: Prof. Dr. Louis Maes
Prof. Dr. Edwin Claerebout

Literature Review
as part of the Master's Dissertation

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FOREWORD

Deze literatuurstudie maakt deel uit van mijn masterproef aan de faculteit Diergeneeskunde van de Universiteit Gent. Voor deze thesis heb ik zelf een onderwerp mogen kiezen en gezien parasitologie mij altijd geïnteresseerd heeft, wilde ik graag een onderwerp binnen dit vakgebied verder uitdiepen. Mijn promotor, Prof. Dr. Louis Maes, en medepromotor, Prof. Dr. Edwin Claerebout, hebben mij bijzonder goed geholpen in het afbakenen van het eigenlijke onderwerp van de thesis. Ik wil hen daarom bedanken voor de begeleiding, goede raad en sturend commentaar tijdens de totstandkoming van dit werk. Verder wil ik ook graag mijn ouders bedanken voor hun onvoorwaardelijke steun tijdens mijn opleiding.

This literature review is part of my Master's Dissertation at the faculty of Veterinary Medicine at the Ghent University. I had the pleasure to choose my own subject for this thesis and since parasitology has always been one of my interests, I wanted to further explore a topic within this field of study. My promoters, Prof. Dr. Louis Maes and Prof. Dr. Edwin Claerebout, have helped me very well in delineating the actual topic of my thesis. Therefore, I would like to thank them for their guidance, good advice and guiding remarks during the development of this work. Furthermore, I would like to thank my parents for their unconditional support during my education.

Michael Meijer

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ENGLISH SUMMARY

Babesia and *Theileria* are haemoprotozoan parasites belonging to the phylum Apicomplexa and are transmitted by ticks. In the tick, sexual reproduction and sporogony take place. During feeding, the sporogony is completed, sporozoites infect host erythrocytes and merogony occurs. In *Theileria*, an intralymphatic cycle precedes the multiplication inside erythrocytes.
Twelve species of *Babesia* have been reported to infect dogs worldwide. Their geographical distribution is largely determined by the geographical distribution of their vector ticks.
In Europe, four species can be found: *B. canis*, *B. vogeli*, *B. vulpes* and *B. gibsoni*. Equine piroplasmosis (EP) is caused by two species: *B. caballi* and *T. equi*, with *T. equi* being more common since horses remain lifelong carriers of this parasite. *B. canis* was considered exotic in the Benelux and Germany. However, in the last few years outbreaks of autochthonous babesiosis have occurred in Northwestern Europe, implying a spread of the infection over Europe. For EP, a spread from more southern endemic countries towards northern non-endemic countries has also been reported.

Dermacentor reticulatus is a three-host tick belonging to the family of the Ixodidae, with adults being active from late August/September through April/May. In our regions, the tick mostly occurs in freshwater tidal marches, providing high soil moisture and sufficient vegetation. Climate change, human activity and changes in ecosystem management have created ideal conditions for *D. reticulatus* to take up permanent residency in areas that were previously unfavorable for the tick.

Piroplasmosis can occur as an uncomplicated form, involving clinical signs directly related to hemolysis, or as a complicated form involving multiple organ failure. The last form is believed to be a result of a derailed systemic inflammatory response syndrome, and is increasingly reported with serious *Babesia* infections in Europe.

Diagnosis of piroplasmosis relies mostly on anamnesis, clinical signs and microscopic examination of blood smears. A variety of additional laboratory tests are available. For horses, the competitive enzyme linked immunosorbent assay is currently the prescribed test by the World Organisation for Animal Health for international horse trading. The most widely used drugs to treat piroplasmosis are imidocarb dipropionate (Carbesia®) and diminazene aceturate (Berenil®). Besides these curative drugs, supportive treatment including IV-fluids, blood transfusions and anti-inflammatory drugs may also be necessary. Prevention of infection is based on vector prophylaxis with acaricides, repellants or manual removal, chemoprophylaxis targeted against the pathogen, vaccination and behavioral prophylaxis.

Key Words: Control – *Dermacentor reticulatus* – Epidemiology – Pathophysiology – Piroplasmosis

DUTCH SUMMARY

Babesia en *Theileria* zijn protozoaire bloedparasieten die behoren tot het phylum Apicomplexa en overgedragen worden door teken. In de darm van de teek vindt de sexuele reproductie plaats. De sporogonie wordt opgestart in de speekselklieren, maar zal overgaan in een ruststadium tot de teek een nieuwe gastheer vindt. Tijdens het bloedmaal wordt de sporogonie vervolledigd en zullen infectieuze sporozoiten de erythrocyten van de gastheer binnendringen, waar ze zich omvormen tot trophozoiten. Deze zullen tweedeling ondergaan en aanleiding geven tot merozoiten. Sommige trophozoiten zullen zich ontwikkelen tot gametocyten om de cyclus te vervolledigen. Bij *Theileria* zal een intralymfatische cyclus de intra-erythrocytaire cyclus vooraf gaan. Naast tekenbeten zijn bijtwonden, bloedtransfusies en het gebruik van met bloed gecontamineerd materiaal ook mogelijke infectieroutes.

Wereldwijd zijn er 12 species van *Babesia* bekend die honden infecteren, waarvan *B. vogeli* het meest voorkomt. De geografische distributie van deze verschillende species wordt grotendeels bepaald door de distributie van de bijbehorende vectorteek of vectorteken. In Europa komen 4 verschillende *Babesia* species voor, namelijk *B. canis*, *B. vogeli*, *B. vulpes* en *B. gibsoni*. In Europa is *B. canis* het meest wijdverspreid, terwijl *B. vogeli* meer voorkomt in het Middellandse-Zeegebied en *B. vulpes* eerder in Noordwest Spanje en Kroatië. Infecties door *B. gibsoni* worden slechts sporadisch gerapporteerd en zijn meestal geassocieerd met de import van geinfecteerde honden uit het buitenland. In de Benelux en Duitsland werd *Babesia* gezien als importziekte. De laatste jaren zijn er echter uitbraken van autochtone *Babesia* infecties gemeld in Noordwest-Europa, wat duidt op een spreiding van de infectie binnen Europa. Al met al blijft de incidentie van caniene babesiose in West-Europa laag, met een percentage van 0,70% per jaar.

Equine piroplasmose (EP) wordt veroorzaakt door 2 pathogenen; *B. caballi* en *T. equi*, waarbij *T. equi* meer voorkomt aangezien paarden na een infectie met deze parasiet levenslang drager zijn. EP is zeer wijdverspreid en slechts enkele landen in de wereld zijn vrij van deze parasitaire infectie. Binnen Europa zijn paardentransporten aan minder strikte regels gebonden en hebben de meeste naburige landen geen geografische barrières die de spreiding van teken zouden kunnen tegengaan. Dit zorgt ervoor dat EP infecties vanuit meer zuidelijker gelegen endemische gebieden naar meer noordelijk gelegen non-endemische gebieden spreiden.

Dermacentor reticulatus is een drie-gastheer-teek welke behoort tot de familie van de Ixodidae. Adulte teken zijn hoofdzakelijk actief van eind augustus/september tot april/mei en parasiteren meestal herten en honden. Zelden zijn mensen het slachtoffer van een tekenbeet van deze species. *D. reticulatus* teken hebben behoefte aan een hoog grondwatergehalte, een droge bodem, veel zon en voldoende gastheren om te parasiteren. In onze regio's komt de teek daarom het meest voor in zoetwatergetijdengebieden, welke een hoog grondwatergehalte en voldoende vegetatie bieden.

Deze tekensoort was vroeger niet aanwezig in de koudere landklimaten van Centraal en Noord Europa. Klimaatverandering, menselijke activiteiten en veranderingen in het ecosysteembeheer hebben echter ideale omstandigheden gecreëerd voor *D. reticulatus* om zich permanent te vestigen in deze gebieden. Ondertussen is deze tekensoort al gesignaleerd in Oost-Duitsland, West-Polen en Tsjechië, wat een spreiding naar het oosten doet vermoeden, maar ook in België en Nederland, wat een spreiding naar Noordwest-Europa impliceert.

Piroplasmose kan voorkomen als een ongecompliceerde vorm, met symptomen die direct toegeschreven kunnen worden aan de hemolyse, of als een gecompliceerde vorm waarbij multipel orgaanfalen kan voorkomen. Deze laatste vorm wordt steeds meer gezien bij ernstige infecties in Europa en men denkt dat deze vorm het resultaat is van een ontspoorde systemische immuunrespons, welke leidt tot een vicieuze cirkel van verminderde orgaandoorbloeding, infectie, ontsteking en necrose.

De diagnose van piroplasmose steunt grotendeels op de anamnese, klinische symptomen en microscopisch onderzoek van bloeduitstrijkjes. De sensitiviteit van microscopische diagnose kan verhoogd worden door het nemen van bloed uit een capillairbed, centrifugatie, het doorzoeken van cellen onder de buffy coat, het gebruik van dikke uitstrijkjes en door te zoeken in de periferie van het bloeduitstrijkje. Immunofluorescence antibody testen, complement fixatie testen, enzyme linked immunosorbent assays en polymerase chain reaction analyses zijn mogelijk als aanvullende laboratoriumtesten. De competitive enzyme linked immunosorbent assay is op dit moment de test die aanbevolen wordt door de Wereldgezondheidsorganisatie voor Dieren om te gebruiken in de internationale paardenhandel.

De meest gebruikte medicijnen om piroplasmose te behandelen zijn imidocarb dipropionaat (Carbesia®) en diminazeen aceturaat (Berenil®). Diminazeen aceturaat heeft echter een lage veiligheidsmarge en kent een grote interindividuele farmacokinetische variatie. Naast deze medicijnen kan een ondersteunende behandeling met intraveneuze vloeistoffen, bloedtransfusies en anti-inflammatoire middelen aangewezen zijn. Preventie van een piroplasma-infectie is gebaseerd op het bestrijden van de vector met acariciden, afweermiddelen of manuele verwijdering, het bestrijden van de pathogeen met preventief werkende geneesmiddelen, vaccinatie en het mijden van gebieden waar veel vectoren voorkomen. Aangezien adulte *D. reticulatus* teken het hele jaar actief zijn, zijn preventieve middelen het hele jaar door aangewezen.

LIST OF ABBREVIATIONS

(c)ELISA	(competitive) enzyme linked immunosorbent assay
APR	acute phase response
ARDS	acute respiratory distress syndrome
ARF	acute renal failure
CARS	compensatory anti-inflammatory response syndrome
CFT	complement fixation test
CIS	Commonwealth of Independent States
CO	carbon monoxide
DIC	disseminated intravascular coagulation
DNA	deoxyribonucleic acid
ECG	electrocardiography
EIA	equine infectious anemia
EP	equine piroplasmosis
EU	European Union
FLP	fibrinogen-like protein
h	hour
IFAT	immunofluorescent antibody test
IM	intramuscular
IMHA	immune mediated hemolytic anemia
IV	intravenous
min	minute
MODS	multiple organ dysfunction syndrome
NO	nitric oxide
OIE	Office International des Epizooties – World Organisation for Animal Health
PCR	polymerase chain reaction
PCV	packed cell volume
PO	per os – oral administration
RNA	ribonucleic acid
SC	subcutaneous
SIRS	systemic inflammatory response syndrome
SLE	systemic lupus erythematosus
SPA	soluble parasite antigens
spp.	species (plural)
TNF	tumor necrosis factor

INTRODUCTION

Piroplasmosis in dogs and horses is caused by two genera of haemoprotozoan parasites, namely *Babesia* and *Theileria*. Twelve species of *Babesia* have been discovered to infect dogs worldwide, of which 4 occur in Europe. In horses, one species of *Babesia* and one species of *Theileria* are responsible for equine piroplasmosis. These organisms are transmitted by multiple Ixodidae ticks. In Europe, the biggest vector of piroplasmosis is *Dermacentor reticulatus*. During the blood meal of the tick, parasites are injected in the host. Asexual reproduction in the host leads to lysis of erythrocytes and may even lead to multiple organ dysfunction and death in severe infections.

The distribution of *D. reticulatus* used to be relatively stable, but in recent years a spread in the range of its occurrence in Europe has been reported. Concurrent with this phenomenon, autochthonous cases of piroplasmosis have recently been seen in regions where this disease used to be exotic. Since the disease is potentially fatal if not diagnosed and treated within a proper timeframe, this evolution is a threat that should not be underestimated.

LITERATURE REVIEW

1. HISTORY OF BABESIA AND THEILERIA

The first report of *Babesia* was made by Victor Babes in 1888[1], who discovered micro-organisms in cattle in Romania showing signs of hemoglobinuria (also named “red water fever”). Unfortunately, he incorrectly assigned these micro-organisms to *Haematococcus bovis*, a name given because these micro-organisms looked like bacterial cocci (“coccus”) found in red blood cells (“Haemato”). He later found similar organisms in sheep erythrocytes. The transmission of this parasite by ticks was confirmed 5 years later by Smith and Kilborne in 1893[2], who also identified the parasite as the cause of Texas fever, a disease similar to red water fever and named the causative agent *Pyrosoma bigeminum*, related to its typical morphology. In that same year, Starcovici[3] united these parasites in the genus *Babesia*, naming them *B. bovis*, *B. ovis* and *B. bigemina*, since the genus name *Pyrosoma* was preoccupied[4]. Since then, other names have been proposed for this genus, the best known being *Piroplasma* by Patton in 1895[5]. *Babesia* presently remains the correct name for the genus.

The parasite description for *Theileria* came later and started with the description of the causative organism of East Coast fever in Southern Africa. This organism was named *Piroplasma parvum* by Theiler in 1904[6]. The fact that this micro-organism was also transmitted by ticks was discovered the same year by Lounsbury[7]. The name *P. parvum* was changed to *Theileria parva* by Bettencourt, Franca and Borges in 1907[8] when they defined the genus *Theileria*. Because of the pear-shaped (pyriform) intra-erythrocytic stages, both *Babesia* and *Theileria* are collectively called “piroplasms”[9,10]. The fact that both babesiosis and theileriosis are commonly grouped together under the name “piroplasmoses” makes that the old genus name *Piroplasma*[10] is still in use. The piroplasms are placed

in the phylum Apicomplexa and consist of the four families Anthemosomatidae, Babesiidae, Haemohormidiidae and Theileriidae[11].

2. THE PIROPLASM LIFE CYCLE

As stated above, *Babesia* and *Theileria* parasites are transmitted by ticks. When a tick feeds on an infected host, infected erythrocytes containing merozoites or gametocytes[12] are taken-up by the vector. The parasites are first detectable in the tick gut after about 10h post-feeding[13] and gametocytes develop into gametes (ray bodies)[12] after about 40-60h[13]. Ingested merozoites will undergo lysis[12]. During the development from gametocyte into gamete, a thorn-like shaped organelle develops at the anterior end of the organism. A zygote is formed upon fusion of two gametes and further develops into a motile kinete (ookinete) which uses the thorn-like shaped structure to enter the epithelial cells of the tick gut[12] at about 80h post-consumption[14].

In *Babesia* species (Figure 1), a cycle of asexual division occurs in the gut epithelium[9], after which the kinetes enter the haemolymph[12] and invade fat body cells and nephrocytes to undergo a second cycle of division, giving rise to secondary ookinetes. These are able to invade the tick ovaries, enabling transovarial transmission[15]. It has to be noted that both primary and secondary ookinetes are able to enter the salivary glands[13]. After infection of the oocytes and/or salivary glands, the parasite becomes dormant[9,13]. Sporogony will further continue in the salivary acini of the next generation larvae (upon transovarial transmission) or the next tick stage after moulting (upon transstadial transmission)[12]. A rise in ambient temperature due to contact of the feeding tick with its host, combined with the consumption of blood[16], will activate the dormant sporoblasts in the salivary acini to develop into infective sporozoites[12]. The sporozoites infect the host erythrocytes and develop into trophozoites, which divide by binary fission into two, sometimes four daughter cells called merozoites. This continuous reproduction cycle results in lysis of the host erythrocyte, liberating merozoites that will enter other erythrocytes and restart the asexual multiplication cycle until the host dies, or until the host immune system intervenes and puts an end to the cycle[10]. Some trophozoites do not divide into daughter cells but develop into gametocytes, which completes the life cycle[12].

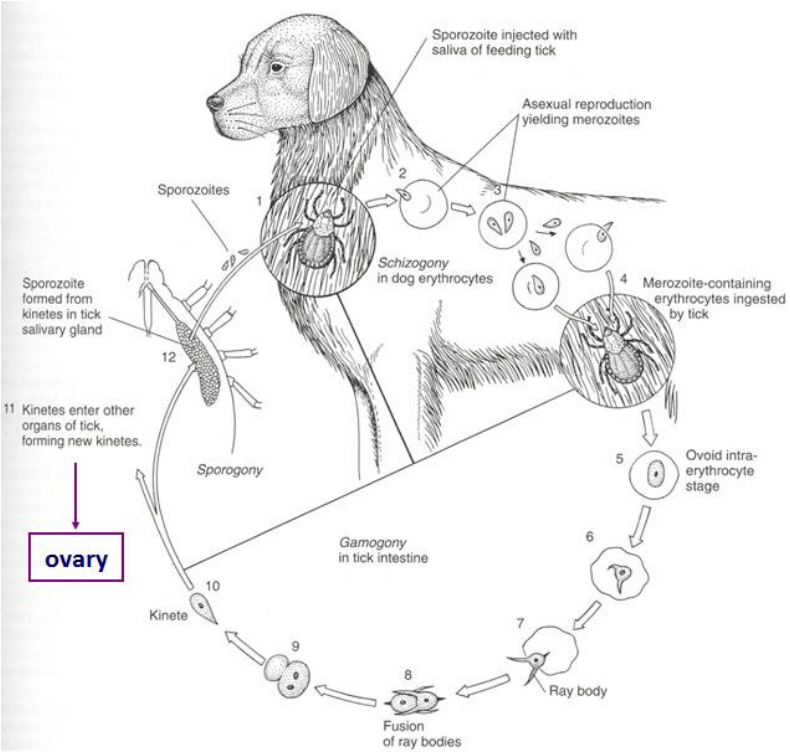

Figure 1. Life cycle of *Babesia*

Image provided by Prof. Dr. L. Maes

In *Theileria* species (Figure 2), the zygotes do not multiply in the endothelial cells of the tick gut, but directly invade the haemolymph where they migrate towards the salivary glands[10]. When the tick feeds on a new host, sporogony and maturation of sporozoites occur. During the final hours of feeding, thousands of infective sporozoites are injected in the dermis along with the tick's saliva[17]. This process establishes infection in the mammalian host[12], whereby infection rates are directly related to the length of the time the tick is attached to the host and which approaches 100% if the tick is allowed to fully complete its feeding cycle[18]. The multiplication inside erythrocytes is preceded by an intralymphatic cycle, where the sporozoite infects a lymphocyte and develops into a schizont. This schizont gives rise to merozoites, which will infect erythrocytes after lysis of the infected lymphocyte. In the erythrocyte, the merozoites develop into trophozoites, which in turn reproduce by binary fission into four daughter cells, creating a "Maltese cross". The further stages in the reproduction cycle in the vertebrate host are the same as for *Babesia* species[12].

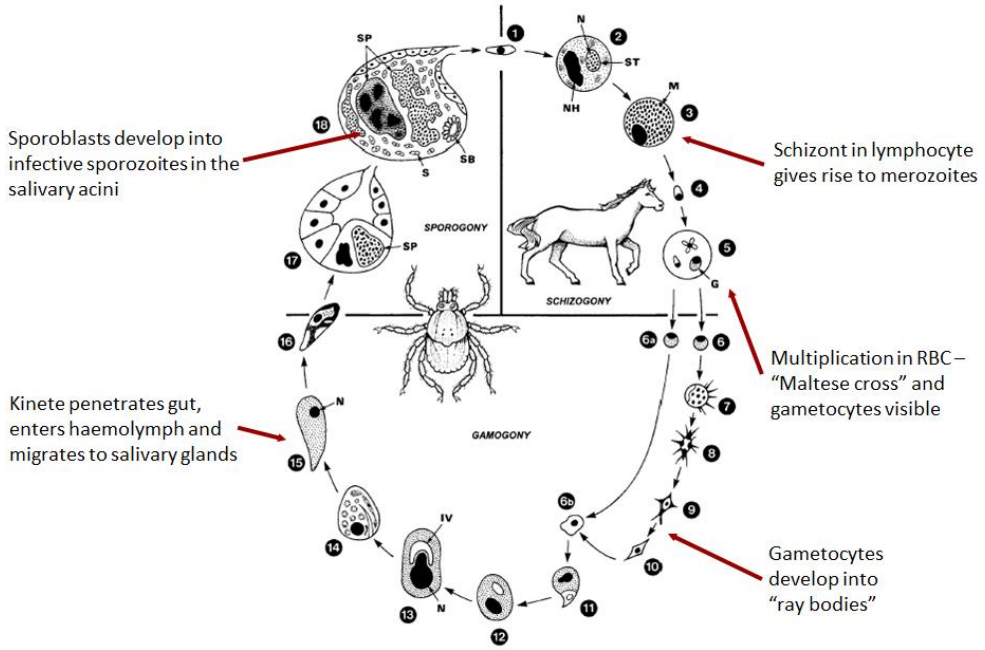

Figure 2. Life cycle of *Theileria*

Image provided by Prof. Dr. L. Maes

3. GEOGRAPHICAL DISTRIBUTION OF CANINE AND EQUINE PIROPLASMOSIS.

3.1 GEOGRAPHICAL DISTRIBUTION OF CANINE PIROPLASMOSIS

Canine babesiosis in Europe was first recorded in Italy in 1895, not long after the detection of this parasite in bovines[19]. Historically, *Babesia* infections in dogs were identified based on morphology of the parasite in the erythrocyte. All large forms (5 x 2.5 µm) were classified as *B. canis*. All small forms (2 x 1.5 µm) were classified as *B. gibsoni*[20,21]. With the availability of molecular diagnostic tools, more piroplasm species infecting dogs were discovered[21,22]. At the time of writing, twelve species of *Babesia* have been reported to infect dogs worldwide[23]. As is to be expected, the geographical distribution of these different species is largely determined by the geographical distribution of their vector ticks[21]. *B. canis* was formerly classified into three sub-species, namely *B. c. canis*, *B. c. rossi* and *B. c. vogeli*. These are now considered to be separate species, because of the variations in geographical distribution, vector specificity, genetic characteristics and the clinical signs they induce in dogs[21,24,25].

The most widespread canine piroplasm is *B. vogeli*, due to the large ecological range of its vector *Rhipicephalus sanguineus*. This piroplasm has a worldwide distribution in tropical and subtropical regions, extending into more temperate zones where it potentially occurs alongside other large *Babesia* spp[21]. In the United States, *B. vogeli* is a well-recognized problem in greyhound kennels[23,26]. Hence, particular care should be taken when relocating greyhounds, since they may harbor subclinical infections. Generally, *B. vogeli* causes mild clinical signs in adult dogs[27]. Parasitaemia also seems to be relatively low, which causes frequent false negative results during routine examination of a blood smear[26]. In puppies however, this parasite may cause severe clinical disease[27].

The most pathogenic species of *Babesia*, *B. rossi*, is an African *Babesia* and was originally reported only in South-Africa. This piroplasm is transmitted by *Haemaphysalis elliptica* (formerly *H. leachi*)[28]. Recently, *B. rossi* has also been reported in other regions of Africa, including Nigeria[29] and Sudan[30], where the vector tick is also enzootic. Both *B. vogeli* and *B. gibsoni* have also been reported in South-Africa[31,32].

B. gibsoni is referred to as the Asian strain of *Babesia*, since it was first reported from a number of different southern, eastern and southeastern Asian countries[21]. Infections primarily occur in the Middle East, southern Asia, Japan, North Africa and South America[26]. The last decade however, *B. gibsoni* infections have been reported in many countries outside of Asia[21]. It is now considered an emerging infectious disease in the United States, and has lately also been detected in Italy, Hungary and Australia[26]. The parasite is seen predominantly in Pit Bull Terrier and Staffordshire Terrier dogs, used for illegal fighting. There is convincing evidence that these new cases of *B. gibsoni* infection are related to biting and fighting between infected and non-infected dogs[33,34]. In much of Asia, *B. gibsoni* infections are not associated with a certain breed of dog, and are naturally transmitted by the vector tick *H. longicornis*[23]. The disease induced by *B. gibsoni* may follow a hyper-acute, acute or chronic course, with the acute course being the most common[26].

In Europe, four *Babesia* species are found, two large and two small (Figure 3). The most widespread is *B. canis*[35] with more than 400.000 dogs per year becoming infected in France alone, emphasizing the widespread nature of this species[26]. *B. canis* is endemic in most of continental Europe and its distribution is closely related with *D. reticulatus*[35]. Its pathogenicity is intermediate between that of *B. rossi* and *B. vogeli* and presents as an acute form, which may occasionally be life-threatening[26,36]. Until now, *B. canis* infections have been reported from Croatia, Poland[37], Hungary[38], Russia[39], Switzerland[40], Germany[24] and France[41].

B. vogeli is found mostly in Southern Europe around the Mediterranean basin[23,36] in the southeast of France, central and southern Italy, Spain, Portugal and Greece[36]. However, it has also been reported outside of this geographical region and is currently considered as an emerging disease in Northern and Eastern Europe[23]. Infection of dogs with both *B.* canis and *B. vogeli* has been reported in Slovenia[42], France[37], Spain[43], Portugal[44] and Albania[45]. In Italy, *B. canis* is mainly found in the northern part of the country, and less frequently in Central Italy. *B. vogeli* is predominantly found in Central and Southern Italy[46].

The two small piroplasms found in Europe are *B. vulpes* (formerly *T. annae*) and *B. gibsoni*[35]. They both cause severe illness and response to treatment is worse[47]. *B. vulpes* was first detected in Northwestern Spain[47] and has since also been detected in Croatia[48] and the United States[33]. *T. annae* was recently reclassified to *B.* vulpes based on phylogenetic analysis and lack of an intraleucocytic stage[49]. Occasional clinical cases of *B. gibsoni* were reported in Spain[50], Germany[51] and Italy[52], primarily as a consequence of the introduction of infected dogs from abroad (mainly Asia, the United States or Australia)[36].

In Spain, several species of piroplasms co-occur according to localization. In Northern Spain, *B. canis* is present, as well as its vector *D. reticulatus*. In Southern Spain, *B. vogeli* is reported alongside its vector *R. sanguineus*. Lastly, the northwestern part of the country is considered hyperendemic for *B. vulpes*. *Ixodes hexagonus*, the suspected vector of this last piroplasm, is also located on the North Atlantic coast[36].

Halos *et al*.[36] found that the overall annual incidence of clinical babesiosis amongst the dog population in Western Europe was 0.70% with large variations amongst countries and amongst regions in each country (0-5.5%). In this study, it was confirmed that babesiosis in France is still widely distributed, with seven regions showing an incidence above 1%. Three main foci were located in South-Western France, reaching the highest incidence (2.4%), continuously linked to a central core including Massif Central (1.1%) and Île-de-France (0.9%) (Figure 4).

Babesia was considered exotic in the Benelux and Germany until recently. In the last few decades, outbreaks of autochthonous babesiosis by *B. canis* have occurred in the Netherlands[53], in the Saarland in Germany[54] and in Norway[55], underlining the spread of infection over Europe. The overall incidence in Northwestern Europe is still low[36], with only some localized occurrences in hotspot areas known to be potentially endemic for *D. reticulatus*. Some sporadic cases observed in areas where the disease was not present were related to imported pathogens in dogs with a history of travel in Southern Europe. In the study by Halos *et al*., 10/13 dogs from the Netherlands had a travel history in Southern Europe. Endemic regions for *B. canis* in Northwestern Europe include the Saarland in Germany[54] and Mons in Wallonia[36].

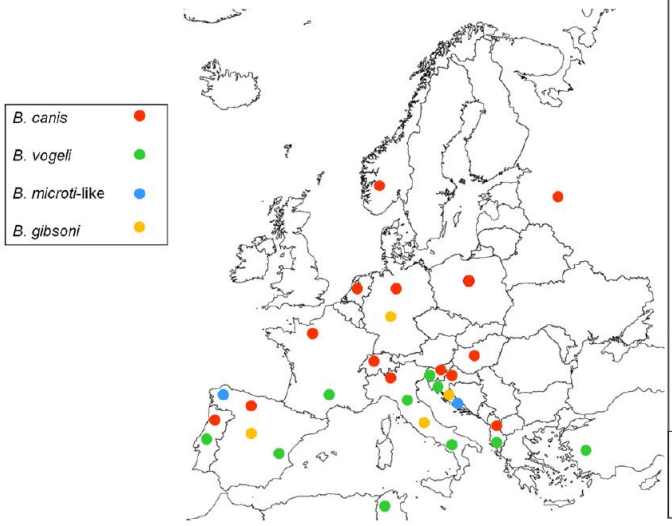

Figure 3. The current geographical distribution of the different *Babesia* species in Europe.
From: Solano-Gallego, L. et al. in Vet. Parasitol. (2011).

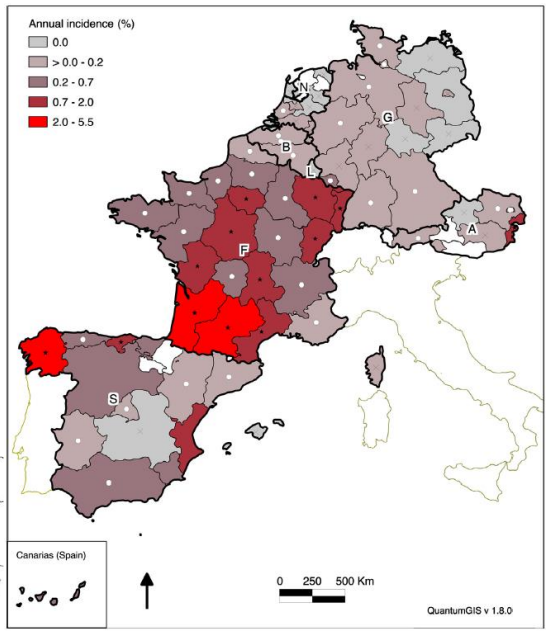

Figure 4. Incidence of canine babesiosis in Western Europe.
From: Halos, L. et al. in Parasite (2014).

3.2 GEOGRAPHICAL DISTRIBUTION OF EQUINE PIROPLASMOSIS

Equine piroplasmosis (EP) is caused by two species: *B. caballi* and *T. equi*[56]. *T. equi* was formerly known as *B. equi*, but was reclassified as a *Theileria* species because of the transstadial transmission in the vector and the presence of a pre-erythrocytic intralymphatic stage in its life cycle[57]. Infections with *B. caballi* usually cause less severe symptoms than *T. equi*[58]. For *B. caballi*, ticks serve as a reservoir due to the presence of transstadial and transovarial transmission in the tick vector. For *T. equi*, only a transstadial transmission is present and thus infected horses are the only reservoir[56,59]. In most regions, infections with *T. equi* are more common than with *B. caballi*[60,61]. This is easily explained by the fact that, once recovered from an acute episode, a horse remains in a carrier state for up to 4 years with *B. caballi* and for life in the case of *T. equi*[62,63].

Few countries in the world are non-endemic for EP. It is estimated that only 10% of the horses globally inhabit regions free of EP[60]. This worldwide prevalence of EP is consistent with the worldwide distribution of competent tick vectors[56]. Both parasites are transmitted by Ixodid ticks of the genera *Rhipicephalus*, *Dermacentor*, *Haemaphysalis*, *Hyalomma* and *Boophilus*[58]. These Ixodid tick vectors occur in tropical, subtropical and some temperate climates[64]. It is important to note that besides tick transmission, iatrogenic means can also be a factor of transmission in case of blood contaminated needles and syringe reuse[65], or blood transfusion[56]. Previous outbreaks in Germany, Switzerland and Australia were already attributed to the use of contaminated needles or instruments[66].

According to the World Organisation for Animal Health (OIE), EP is endemic in Southern Europe (Portugal, Spain, France and Italy[60]), Asia, countries of the Commonwealth of Independent States (CIS, southern parts of Russia), Africa, Cuba, South and Central America and certain parts of the southern United States of America[67]. The situation in Europe during the first half of 2015 is shown in figure 5. *T. equi* has also been reported from Australia, but apparently never established itself in this region[67]. Most infections of horses in non-endemic European countries have been traced back to Spain, France, Italy or the CIS[60]. In endemic regions, most horses are exposed to EP within the first year of life. Case fatality rates of 5-10% are reported in naïve horses, depending on the species, the transmission dose, health of the horse and treatment. If infected horses are imported into regions where naïve horses are present, fatality rates may exceed 50% depending on the amount of vectors and infected horses present[68,69].

In continental Europe, where most neighboring countries have no geographical barriers and horse movements are free, there is a trend for EP to move from endemic countries towards northern non-endemic countries, for example Belgium[70], Switzerland[71] and more recently the Netherlands[72]. In a study conducted by Guidi *et al.*[58] a seroprevalence of 58% for *T. equi* and 12,9% for *B. caballi* was found in the Camargue region in France. In addition, 8.1% of the tested horses were positive for both species. Other studies reporting seroprevalences in Europe are scarce, and seroprevalences may vary according to the diagnostic technique used. In Switzerland, the overall seroprevalence for EP was 7,3%[71]. In the province of Zeeland in the Netherlands, a seroprevalence of 4% was reported[72]. In Greece[73] and Portugal[74], seroprevalences ranged between 11-17,9% for *T. equi* and 2,2-11,9% for *B. caballi*. In central (Hungary[75]) and southern (Spain[76] and Italy[77]) Europe, seroprevalences were found to vary much more, ranging from 32-68%. Overall, these numbers indicate that Mediterranean countries are at much higher risk of EP than the Northern regions in Europe. However, autochthonous EP has been confirmed in the Netherlands[72] and Belgium[70], albeit sporadically. With the establishment of indigenous *D. reticulatus* populations[78] (see below) and unrestricted importation of horses from piroplasmosis endemic areas, the chance of encountering clinical EP cases in these northern countries is likely to increase.

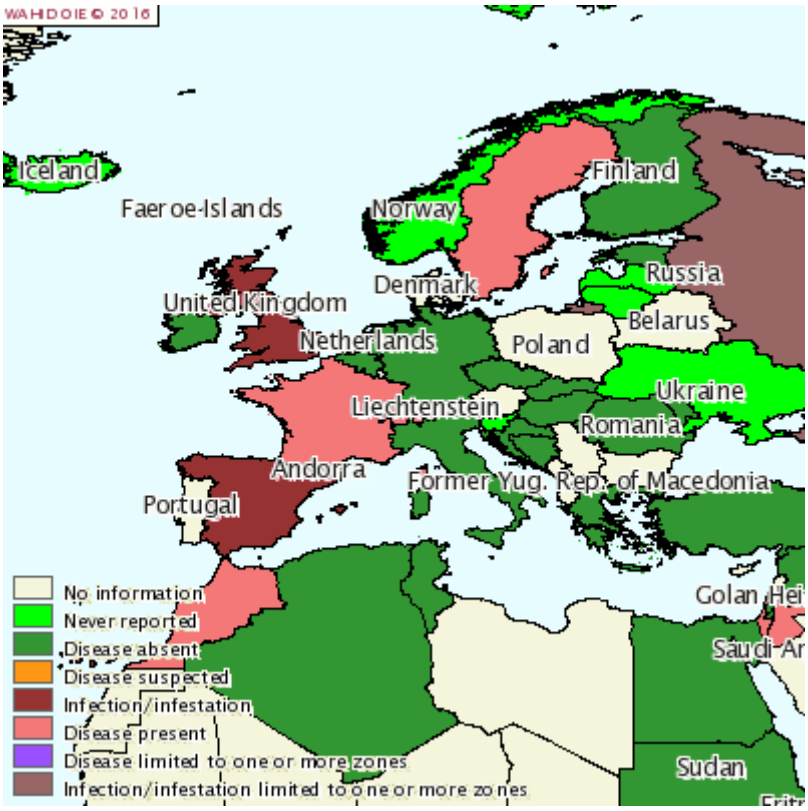

Figure 5. Reported cases of EP in Europe from January to June 2015

Image taken from the WAHIS Interface from the OIE website (www.oie.int)

4. THE VECTOR *DERMACENTOR RETICULATUS*

4.1 LIFE CYCLE

Dermacentor reticulatus is a hard tick belonging to the genus *Dermacentor*[79]. Similar to most other species of the family *Ixodidae*, it is a three-host tick where each stage feeds on another host to complete its life cycle[80]. Adults are mainly active from late August/September through April/May[81], with a peak abundance in Belgium between March and April[82]. In winter periods, the low temperatures and potential snow cover interrupt their questing behavior[81]. Most adult ticks are inactive during the summer months[78,83]. Copulation takes place on the host, after which the adult female feeds for about 9-15 days[84]. Egg laying occurs 3-4 days after the fully engorged female has dropped off the host, after which the adult female dies[85]. Oviposition exclusively takes place in spring[81] and lasts for 6-25 days during which 3000-4500 eggs per female are laid in a sheltered spot. The development in the egg takes 14-21 days and the larvae need 2-6 days for a blood meal[80] with a main activity period in July[81].The molting process on the ground takes 14 days and results in a nymph, which feeds for about 5 days[80] with a main activity period in August[81]. Larvae and nymphs do need a resting period of 2-4 weeks after emergence from the egg or molting, before they start feeding[85]. After dropping from the host, the nymph molts into an adult which may take 12-14 days. The adult can starve for up to 1.5 years before finding a new host and completing its life cycle[80].

The development times of the different tick stages are influenced by environmental conditions such as temperature and humidity[84,86]. When development times are short and the different stages are successful in finding hosts, several genera may complete within one season, resulting in rapid spreading[80].

The main hosts for larvae and nymphs are small mammals and rodents, while adults mainly parasitize larger mammals like deer and dogs[78,85,87], and only occasionally humans[81,87].

4.2 HABITAT AND GEOGRAPHICAL DISTRIBUTION

Since adult *D. reticulatus* ticks quest on vegetation, it may occur in quite different habitats ranging from freshwater tidal marches[78,88] to urban wastelands[89]. Typical biotopes include marshes, meadows and shrub pasture communities[87]. The tick can also be found in inner city parks[87,90–92], typically those which have been created from natural meadows and forests and closely resemble their natural habitat[87]. Although a great variety of possible biotopes for *D. reticulatus* have been reported, there are some general features which make a habitat particularly favorable. First of all, a combination of high level of ground water along with a drying soil[78,87,93]. Secondly, the amount of solar radiation has to be relatively high[94], which explains why the tick does not occur in shady forests[89,94]. Lastly, hosts must be present, which explains why habitats for *D. reticulatus* are all characterized by the presence of free-ranging populations of small and large ruminants[78]. In the Netherlands and Belgium, *D. reticulatus* ticks were found to be most abundant in freshwater tidal marches, all situated in close proximity to waterways. These habitats provide high soil moisture and the present vegetation provides sufficient shelter against environmental changes. Hence, large host populations can be present in these habitats[78,88]. Other localities in the Netherlands include moist dune valleys, fallow land and a moist deciduous forest, although these locations appeared to be less favorable areas to survive[88].

D. reticulatus is also found in the temperate climates of Eurasia, where its occurrence has been divided in an eastern and western part[95], because the tick used to be relatively rare or absent in the cold continental climates of Central and Northern Europe[96]. The reason for this eastern and western division of tick occurrence is not exactly known, but hypotheses include different environmental and climatic conditions between these geographical zones, as well as human activity[87]. Given that factors such as ambient temperature, humidity and insulation levels have an effect on the development of the different stages[97], the occurrence or absence of this tick becomes highly dependent on microclimatic conditions. For example, an explanation for the absence of *D. reticulatus* in Central Europe may be given by the particular characteristics of the winter season. The eastern part of the division is characterized by a thick snow cover in the winter providing a perfect insulating protection allowing stable overwintering and survival in safe places on the soil surface. This snow cover is not sufficient to protect ticks in Western Europe, but ticks can nevertheless overwinter since the number of frost days is low. In Central Europe, the thin snow cover does not allow protection against frost, and hence explains the absence of the tick in this area[87].

The distribution of *D. reticulatus* described above has remained relatively stable, however, some recent reports mention new occurrence sites where *D. reticulatus* was previously absent. In Germany, a study in 1976 reported *D. reticulatus* only at 4 sites out of more than 3000 while a more recent study reported 40 sites, clearly showing an expansion to the east[94], which has also been confirmed by the finding of new sites in Western Poland[95] and the Czech Republic[98]. In addition, recent reports of indigenous *D. reticulatus* populations in Belgium and the Netherlands confirm its spread in northwestern Europe[78,82,88,99], since the French-Belgian border used to represent the northern boundary of *D. reticulatus* in Western Europe[82].

The current geographical distribution of *D. reticulatus* in Western Europe (Figure 6) ranges from northern Spain and Portugal through France and Belgium up north to the Netherlands and southwest England[100]. The occurrence of *D. reticulatus* in Italy is very rare[101]. In Eastern Europe, the southern border ranges from Central and Eastern Croatia[100], through Belgrade in Serbia[102] to the Crimean Peninsula in Ukraine[100]. The tick occurs as far east as the basin of the Yenisei river in Siberia. The most northern limit of the eastern distribution area is in the southeast of Latvia[103].

The more northern limit in Eastern Europe as opposed to Western Europe may be caused by warmer summers in Eastern Europe because of the more continental climate, which allows more ticks to complete their development in one growing season[100]. *D. reticulatus* is absent in the entire region of the European Alps, presumably caused by the cooler temperatures associated with greater height. These cooler temperatures increase developmental periods of the tick and make for fewer available hosts in this geographical region. However, the tick is frequently found at the northern periphery of the entire European Alpine arc. Since *D. reticulatus* prefers temperate climates it does not occur in the Mediterranean climatic zone[100]. Within geographic regions of occurrence, *D. reticulatus* is distributed in a highly focal pattern associated with its particular habitat needs[94,97].

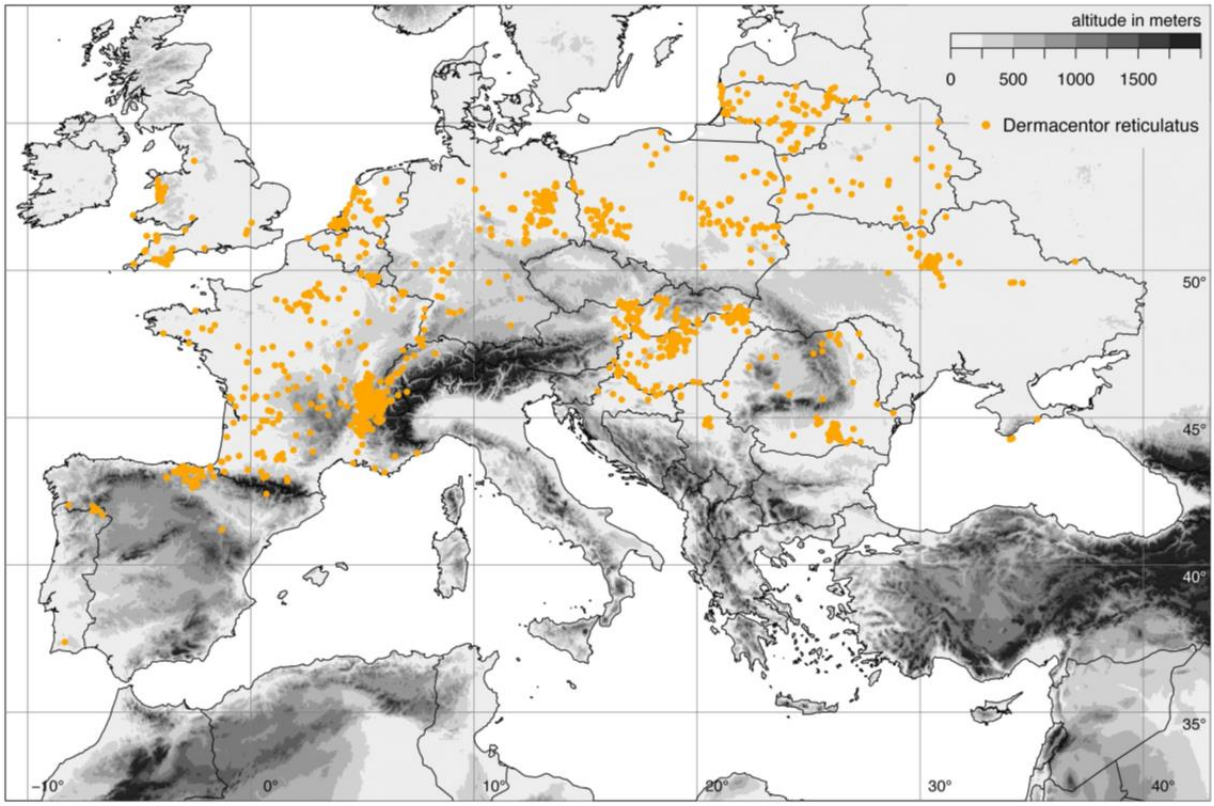

Figure 6. The current geographical distribution of *Dermacentor reticulatus* in Europe.
From: Rubel, F. et al. in Ticks Tick. Borne. Dis. (2015).

4.3 REASONS FOR THE RECENT CHANGE IN DISTRIBUTION

The reasons for the recent spread of *D. reticulatus* in Europe are still unclear, but current hypotheses can be divided in two groups: natural factors (associated with climatic changes) and factors associated with human activity (such as migration and travel)[104]. Most hypotheses refer to global warming, but many other factors definitely impact on the epidemiology of *D. reticulatus*[105]. Furthermore, *D. reticulatus* has a high ecological plasticity, so climate warming does not seem to be the major direct factor responsible for its recent geographical expansion[106]. In contrast to this statement, the threshold temperature and humidity for the activity of *D. reticulatus* are relatively low, meaning this tick species would be particularly susceptible to changing climatic factors[87].

4.3.1 Global warming

Climate change is no longer debated and certainly has an impact on environmental factors responsible for tick survival. In Western and Northern Europe, climate change leads to shorter and less severe winters[105], extended spring and autumn seasons[86] and wetter summers[105]. This means an increase in mean winter temperature, a decline in the number of frost days and the earlier appearance of snow cover with shorter persistence[87,107]. The warmer climate especially favors the overwintering of eggs and larvae that otherwise would not survive the cold winter in Central Europe[87,108] and prolongs the activity period of adults[87]. In Belgium and possibly also other countries with more or less similar

climatic conditions, a higher number of adult *D. reticulatus* ticks is found during the spring activity peak. This observation may imply that climatic factors during the winter diapause have a definite effect on maintaining tick population levels[82,106]. The shorter "classic" winter period also allows ticks to be active all year round[96]. In the Netherlands, ticks were found throughout the year, although very few ticks were seen during the summer months[78]. In Poland, adults were found on dogs as late as December after the appearance of snow cover[109]. Another consequence of this changing climatic factor is the decrease of the length of snow cover, negatively impacting tick winter survival but allowing roe deer (the primary host of *D. reticulatus*) to find more food[86]. Not only milder winters exert their effect on tick biology. As stated above, habitats harboring adult *D. reticulatus* ticks are all characterized by more or less intense solar radiation. Different processes in the life cycle take place at the soil surface, such as oviposition and the development to different life stages. The soil surface temperature has to be above the developmental zero to guarantee completion of development. Therefore, the temperature sum (cumulative day-degrees above the developmental zero) at the soil surface is a limiting factor for these processes[94]. Warmer summers may allow increased rates of development, or make development possible in places where this was not possible earlier[86].

Apart from the effect on the mean temperature and change in seasons, global warming is also associated with acute phenomena, such as floods and storms. These phenomena may also improve local conditions for tick survivability[96].

All things considered, it can be concluded that the effects of climate change on tick epidemiology are incredibly complex and not always straightforward. As of now, changes in tick epidemiology and therefore the epidemiology of vector-borne diseases are difficult to predict. Further studies should focus on enhancing possibilities of modeling these changes, so that measures can be taken to prevent the spread of vector-borne diseases.

4.3.2 Human activity

Besides climate change, human activity[87,94,105,106] and changes in ecosystem management[96] can also influence the dynamics and geographical distribution of *D. reticulatus*. In Central and Eastern Europe, the reform of politics after the fall of the Soviet rule caused changes in agricultural practices, local reforestations and a reduction in applied pesticides and the number of cattle[87]. These may directly influence habitat parameters crucial for tick survival[110].

The European Union supports the closure of agricultural areas and large portions of agrarian soil transform into fallow land, meadows or wooded land[94,97]. Arable land features drastic changes in plant cover and soil management each year and is therefore less suitable for ticks. Fallow land features more stable conditions and high plant cover; and is therefore a more suitable habitat for *D. reticulatus* and its hosts, such as roe deer, foxes, small mammals and rodents[110]. Most of these closed agricultural areas are transformed into semi-natural reserves[88,96]. The presence of dense vegetation in

these protected zones ensures that the conditions are relatively stable and isolated from extremes in temperature, humidity and other meteorological factors[87,110]. Moreover, reconstituted populations of large mammals in these reserves also provide opportunity for ticks to feed on and be transported to new areas. By protecting and restituting the population of wild large mammals and birds, man has created ideal conditions for *D. reticulatus* to take up permanent residency[88].

Another form of environmental protection is the creation of strips of land, isolated from human activity, to connect different pieces of nature or protected natural areas (habitat connectivity initiatives, sometimes called “deshredding”). The creation of such ecological corridors is favorable for the expansion of many animal species, amongst them ticks[86,87]. An example of such an ecological corridor is the newly built ecoduct “Kempengrens” near the border between the Netherlands and Belgium, connecting wood- and heathland on both sides of the A67/E34 highway. This ecoduct now allows deer, weasels and foxes to freely move between these two ecozones[111].

Dense vegetation and rich flora is also present in urban parks and other green terrains and these are therefore a suitable habitat for many species of mammals, birds and invertebrates. For wild animals to settle in or migrate to these parks, it is important, that these parks are in close proximity to natural forest complexes on the outskirts of town[87]. There have been repeated problems of deer and ticks in residential gardens due to urban green corridors in the United Kingdom, suggesting that migration of wild animals and ticks to urban areas is definitely a possibility[112]. These animals may carry attached ticks, which in their turn can settle in the park environment. It is also possible that city dwellers travel to *D. reticulatus* endemic areas and bring back ticks on their pets to new locations in urban sites, such as parks and other recreational areas[53,110].

The influence of human activity on the dynamics and geographical distribution of *D. reticulatus* is not limited to changes in ecosystem management. Increased tourism, transport of animals and movement of people between countries also contributes to the spread of ticks[87,96]. As stated above, people who travel with their pets to endemic areas may bring back engorged *D. reticulatus* ticks, which may form indigenous populations in the new habitat. Also, large animals transported for trade or farming may be parasitized by *D. reticulatus* ticks before transport, and detach at the place of arrival. Due to this fact, *D. reticulatus* already migrated from France to the United States via transport of horses and other animals in the 1960s, 1970s and 1980s[113].

5. PATHOGENESIS AND CLINICAL SIGNS

The incubation period for piroplasmosis after a tick bite is 1-3 weeks[114,115] and the primary effect of infection is the destruction of erythrocytes, resulting in anemia. Uncomplicated piroplasmosis involves clinical signs directly related to the hemolysis[114,116,117], such as pale mucous membranes, icterus, splenomegaly, water hammer pulse, hypotension and hemoglobinuria[118–121]. However, complications involving multiple organ failure may develop, resulting in symptoms that are not easily explained by the hemolytic disease process alone. This is named complicated piroplasmosis and may include acute renal failure (ARF) / renal damage, hepatopathy, immune mediated hemolytic anemia (IMHA), pulmonary edema / acute respiratory distress syndrome (ARDS), disseminated intravascular coagulation (DIC), rhabdomyolysis and cerebral dysfunction. This complex coherence of symptoms is believed to be a result of a derailed systemic inflammatory response syndrome (SIRS), leading to multiple organ dysfunction syndrome (MODS)[28,114,117,120] . Complicated babesiosis is associated with virulent *B. rossi*, but is increasingly reported in association with serious *Babesia* infections in Europe[23]. Clinical signs may develop peracute, acute or chronic and symptoms can also be rather vague, including lethargy, weakness, poor performance, vomiting, anorexia and fever[23,28,56]. Clinical manifestations of the complicated form depend on the particular organs involved. Regardless of treatment, a chronic phase (state of premunition) develops in most cases. This state may be linked to the sequestration of parasitized erythrocytes in particular organs[23,122]. Asymptomatic carriers are also reported. The low level parasitaemia in these animals is undetectable on blood smears, but they do represent a transmission risk and are at risk of developing clinical piroplasmosis in case of concurrent disease or stress (*e.g.* anaesthesia or strenuous exercise)[56,114,123]. The pathogenicity is determined by the species and strain involved, the age of the host and the immunological response generated against the parasite[28,114,116,121]. IMHA and systemic lupus erythematosus (SLE) are diseases that should be differentiated from piroplasmosis[114]. In horses, equine infectious anemia (EIA) and purpura hemorrhagica should be added to the differential diagnosis[56]. Vertical transmission of *Babesia* and *Theileria* is possible and has been reported in dogs[123] as well as in horses[56,124]. Puppies are protected by colostral antibodies until 6 weeks[123]; foals until 4-5 months[125,126].

More and more researchers believe that a uniform mechanism leads to the different clinical manifestations seen during piroplasmosis. This is based on the hypothesis that SIRS is a pathophysiological mechanism that underlies the different presentations of both uncomplicated and complicated piroplasmosis, since both of these disease forms are associated with host inflammatory responses[28,114,117,120,127]. SIRS is the name given to the clinical manifestation caused by the effects of intrinsic mediators of the acute phase reaction (APR)[128,129]. An animal is considered as having SIRS if two or more of the following clinical signs occur: tachycardia, tachypnea (or respiratory alkalosis), hypothermia or hyperthermia, leukocytosis or leucopoenia or neutrophilic left shift[127]. It should however be noted that when a pro-inflammatory state is induced, anti-inflammatory mediators (compensatory anti-inflammatory response syndrome, CARS) are also initiated. The intensity of SIRS and CARS should be balanced and proportional to the insult. Different factors (such as genetics,

environment and constitution) may lead to an unbalanced reaction in favor of SIRS, possibly leading to MODS[116].

The anemia, which is the hallmark of piroplasmosis, is caused by multiple factors. Firstly, parasitaemia results in osmotically fragile erythrocytes, hemolysis, and subsequent anemia[114]. However, the severity of the anemia is not proportional to the low degree of parasitaemia usually observed (<1%). This indicates that additional factors are involved and that not only parasitized cells are destroyed[114,117,130]. Secondly, infected erythrocytes incorporate parasite antigens into their surface, inducing host antibodies that opsonize the erythrocytes, leading to removal of infected erythrocytes by the mononuclear-phagocyte system in the spleen and the liver[114,116,131]. The hyperplasia of the mononuclear-phagocytic system may lead to splenomegaly[114]. Erythrophagocytosis of non-parasitized red blood cells also occurs[131]. Thirdly, activation of the kallikrein system by soluble parasite proteases induces fibrinogen-like protein (FLP). This makes the erythrocytes stickier, leading to “sludging” in the capillaries. This phenomenon is thought to contribute to the acute anemia. The brain and muscles appear to be most severely affected by the sludging[114]. Hypotension (see below) can facilitate or potentiate the stasis[132]. Sludging in the brain leads to localized endothelial injury, followed by necrosis, perivascular edema, microhaemorrhages and ultimately malacia[121]. This may induce cerebral babesiosis, which is clinically defined as babesiosis with neurological signs that cannot be attributed to any other cause. These symptoms can include incoordination, hindquarter paresis, nystagmus and muscle tremors[114,117]. In muscles, sludging may cause rhabdomyolysis, which could play a role in piroplasmosis-associated renal failure[133]. Fourthly, oxidative stress is a possible cause of erythrocyte damage, especially the erythrocyte membranes, resulting in lysis[116], increased susceptibility to phagocytosis and increased rigidity of erythrocytes, slowing their passage through capillary beds[114]. Lastly, complement-mediated destruction of both parasitized and non-parasitized erythrocytes is also an important aspect leading to anemia[114,117]. Free myoglobin (due to rhabdomyolysis) and free hemoglobin (due to hemolysis) may play a role in the renal complications seen during *Babesia* infections, but this remains controversial[56,133–135]. A decrease in hematocrit may also be caused by the dilution of blood due to hypotension. Hypotension increases water retention, thus diluting cellular blood elements and plasma factors[120].

Upon infection, the onset of the APR is dependent on the infectious dose[120]. Fever is caused by the APR itself, the release of soluble parasite antigen (SPA) when the parasites escape from red blood cells, and by the release of (altered) red blood cell components during erythrocyte lysis[114,117,120]. The major mediators of the host inflammatory response are cytokines, nitric oxide (NO), free oxygen radicals, eicosanoids and platelet activating factor[127,136]. Cytokines (Tumor Necrosis Factor (TNF), Interleukin-1 and Interleukin-6 for instance) are produced during infection or trauma and induce the effector molecules of SIRS. They are important in the host defense against the parasite, but excessive or unbalanced production is detrimental to the host[117]. These cytokines induce inflammation in multiple organs, including the heart[137] and kidney[138], and also increase vascular permeability. This last effect causes extravasation of fluids from the intravascular to the extravascular compartment, inducing

hypotension[114]. This hypotension is further enhanced by NO, a secondary mediator induced by TNF inducing vasodilatation, and myocardial damage[114,117,139]. Free oxygen radicals are generated by phagocytes as a defense mechanism against infections, but may also contribute to host cell damage[117]. These compounds also increase inflammatory cell chemotaxis and activity[140]. The increased vascular permeability and myocardial dysfunction, increasing hydrostatic pressure in the pulmonary veins, predispose for ARDS and associated pulmonary edema. This is a severe and frequently catastrophic complication of babesiosis[114,117,121]. ARDS is characterized by tachypnea, dyspnea, moist cough and blood-tinged frothy nasal discharge[117]. In case of oliguria (see below), iatrogenic fluid overload may also cause pulmonary edema[141]. The extravasation of fluids may also induce a paradoxical phenomenon called “red biliary”. Red biliary is characterized by congested mucous membranes, despite the presence of grossly visible haemoglobinemia and/or haemoglobinuria (indicating hemolysis), and a high-normal or elevated packed cell volume (PCV)[114,117,131]. These signs are a strong evidence for an overall increased vascular permeability, which has important and worrisome implications for fluid therapy[121].

Renal involvement or ARF in piroplasmosis is thought to be caused primarily by the hypotension, potentiated by hemoglobin casts, acidosis, compounds from lysed blood cells, the inflammatory mediators itself[117,142] and the redistribution of blood flow due to myocardial damage[121]. Renal failure includes symptoms of anuria or oliguria, despite adequate rehydration. Furthermore, proteinuria, casts and renal tubular epithelial cells are present on urinalysis and blood analysis reveals azotemia[114,117].

The combination of hypotension, hemolysis, vascular stasis and myocardial damage causes hypoxia in most, if not all, tissues[28,114,117], increasing the inflammatory processes already present. This hypoxia is enhanced by excessive endogenous CO production due to the increased haem catabolism, further impairing oxygen transport[114,143]. Centrolobular cells of the liver are highly susceptible to ischemia[144], hence, centrolobular congestion or necrosis is not uncommon in *Babesia* or *Theileria* infections[56,114,117,121,142]. This hepatocellular damage and bile stasis can lead to icterus, elevated liver enzyme levels and elevated bile acid levels[114,117]. Acute pancreatitis may also be explained by pancreatic ischemia[142], leading to icterus, vomiting, melena, abdominal pain and diarrhea. Myocardial ischemia, in combination with myocarditis, may result in arrhythmias seen on ECG[121]. Besides these complications, the tissue hypoxia will obviously increase the lactic acid production by anaerobic glycolysis. This phenomenon induces metabolic acidosis[114,117], which increases pulmonary vascular resistance and reduced pulmonary function, further enhancing ARDS[114]. Respiratory alkalosis is also seen in infected animals, resulting partly from compensation, but more directly from hyperventilation caused by the hypoxemia[114,121].

Impaired gluconeogenesis and decreased glycogenolysis (caused by a depletion of hepatic and muscle glycogen stores) may lead to hypoglycemia[145,146]. Hypoglycemia can be the cause of coma, collapse and other neurological signs and is thus an important differential diagnosis for cerebral babesiosis[114,142].

Because of the intense stimulation of the immune system, IgG and IgM antibodies and complement may keep attacking erythrocyte membranes (recognized as foreign), despite successful antiprotozoal treatment. This process is called secondary IMHA and results in intravascular agglutination, followed by erythrophagocytosis[56,114,121,147]. The contribution of the immune system to erythrocyte destruction (discussed earlier) should however not be confused with secondary IMHA[117]. It is important to identify clinically important IMHA to treat it aggressively and timeously, while avoiding immunosuppressive drugs in animals that do not require them[121].

Besides the obvious involvement of red blood cells in the pathogenesis, thrombocytes are also affected during infection. Fibrinogen, which increases in plasma during the APR, coats platelets (by binding to the GPII/IIIa receptor), which leads to platelet aggregation and activation of the coagulation system[120]. This could be one of the factors explaining DIC in infected animals, apart from hemolysis, acidosis, hypoxia and shock, which are all known predisposing factors for DIC[28,114,148]. DIC leads to microthrombi. These thrombi cause further capillary ischemia and local organ damage, particularly in the kidneys, lungs, liver, gastrointestinal tract and central nervous system[148]. Sustained activation of the coagulation cascade leads to a shortage of coagulation factors, resulting in an increased coagulation time[120] and bleeding tendency[148]. Apart from fibrinogen, induced plasma kallikrein levels can also activate the coagulation cascade at factor XII[114].

In conclusion, fulminant SIRS can lead to MODS through a sequence of hypoperfusion, infection, inflammation and necrosis[127]. If the complications are severe enough, shock, collapse and death are a possible result[114,120,121,142]. It should be noted that, even in animals with piroplasmosis presented in a state of collapse, classical shock symptoms may not be present. Pulse may be bounding or weak, temperature elevated or subnormal, mucous membranes may be pale or congested (in case of red biliary). It is possible that the shock seen in piroplasmosis passes through a hyperdynamic stage, followed by hypotensive shock[117].

6. DIAGNOSIS, TREATMENT AND CONTROL

6.1 DIAGNOSIS

Since no testing procedure for piroplasmosis offers a 100% certainty of detecting an infection, a combination of different techniques may be necessary. These include: anamnesis, including history of travel in endemic areas and tick infestation, blood transfusion or other possible non-vector-borne transmission routes, clinical findings and laboratory abnormalities, direct detection in blood smears stained using Giemsa, Wright or Diff-Quick or DNA detection by polymerase chain reaction (PCR), and indirect detection by serology using a (competitive) enzyme-linked immunosorbant assay ((c)ELISA) or an immunofluorescence antibody test (IFAT)[149].

Microscopic examination remains the simplest and most accessible diagnostic test for most vets and in acute disease it is reasonably sensitive, provided that blood films are well prepared and suitably stained[21,23]. Sampling from capillary beds like the ear tip of the patient or examination of cells beneath the buffy coat of a hematocrit tube may improve the probability of finding parasites[27]. Since parasitized red blood cells tend to marginate during the making of the smear, searching along the periphery of the blood smear also increases the likelihood of finding parasites[26]. Lastly, thick blood smears as well as centrifugation both significantly increase the number of erythrocytes to be screened and may help to increase sensitivity[69]. In chronic or subclinical piroplasmosis, where parasitaemias tend to be very low, sensitivity of microscopy is very low. Furthermore, species or genotype of the organism cannot be determined by morphology alone[23].

In IFAT, parasite antigens are bound to a glass slides with a fluorescein-labeled anti-equine or anti-canine serum and allowed to react with test sera. Bound antibodies are visible under UV-light. For equines, sera are considered positive if strong fluorescence is obtained at a dilution of 1:80 and higher[56]. For canines, the same cut-off value has been established for *B. canis* infections. A cut-off titre of 1:320 or greater has been established for incriminating *B. gibsoni* infections[114]. It is important to note that very young animals or animals tested early in the disease course may be serologically negative[114]. Antibodies remain detectable during the latent period of infection[56], which may be problematic for clinicians working in endemic regions. Disadvantages of this technique are the need to produce large amounts of antigen, cross-reaction between *Babesia* spp. and other apicomplexan parasites, time consumption of the test and the fact that it is difficult to standardize due to the subjectivity in the interpretation of fluorescence[21,69,150].

In horses, a complement fixation test (CFT) has also been developed. CFT was the previous official test for EP, but has now been replaced by the more reliable cELISA. CFT is based on the complement fixation during the reaction between specific antigen and antibody, and is considered positive when the reaction is positive at a dilution of 1:5. Disadvantages of the CFT are the need for production of large quantities of antigen, false negative results, cross-reactivity and low sensitivity in chronic cases[56].

The cELISA is currently the prescribed test by the OIE for international horse trading[67]. It detects antibodies to both *B. caballi* and *T. equi* using specific monoclonal antibodies, and has demonstrated improved performance compared to CFT and IFAT, especially in cases of inapparent carriers[69]. cELISA tests are available as kits for detection of antibodies to either *B. caballi* or *T. equi*[56]. ELISA and dot-ELISA tests have been developed for the diagnosis of canine piroplasmosis as well, but are used more for seroepidemiological studies than clinical diagnosis[114].

PCR is a highly sensitive diagnostic tool, but it is currently mainly used for research purposes and not routinely available for clinical diagnosis[21,60]. Lately, the test is becoming more affordable and it may soon become commercially available. It is important to note that PCR will clearly not detect target DNA when there are no organisms in the sample, which may be the case in chronically infected animals. This may lead to "false negative" results, and is important to recognize when screening potential carriers and other asymptomatic animals such as blood donors[21,23]. In these cases, the use of serology as an alternative, complementary diagnostic test is advisable[21,150] . Another advantage of PCR is that, following a positive result, species differentiation can subsequently be performed by either species-specific real-time PCR or small subunit ribosomal RNA amplification with subsequent sequencing[150].

6.2 TREATMENT

The most widely used drugs to treat piroplasmosis are imidocarb dipropionate and diminazene aceturate. The therapeutic strategy consists of stopping multiplication of the intraerythrocytic parasite and to limit the harmful consequences of the infection, while allowing persistence of several parasites in order to induce immunity[151]. Besides curative drugs, supportive treatment including IV-fluids, blood transfusions and anti-inflammatory drugs may be necessary[23,151].

6.2.1 Treatment in dogs

In dogs, a single injection of imidocarb dipropionate (Carbesia®) at a dose of 2,125 mg/kg IM is used to treat large forms of *Babesia* spp. Most dogs show a good clinical response within 48h after treatment[22,151]. The injection is painful and irritating, but not necrotic. Vomiting 15 min following injection is another common side-effect, and may be accompanied by colic, diarrhea and mild ptyalism[151]. Pretreatment with a parasympatholytic drug, such as atropine or glycopyrrolate, may be appropriate to minimize these side-effects[56]. Diminazene aceturate (Berenil®) is used to treat large and small *Babesia* spp[22] at a dose of 3,5 mg/kg IM or SC, but has a relatively small safety margin with a large inter-individual pharmacokinetic variation[152]. Clinical and parasitological cure are commonly not achieved in infections with small forms of *Babesia* spp, and clinical relapses may occur frequently in these infections[22]. *B. gibsoni* is particularly resistant to treatments with imidocarb dipropionate and diminazene aceturate. An alternative therapy consists of a combination of atovaquone (13,3 mg/kg every 8h PO) and azithromycin (10 mg/kg every 24h PO) for 10 days[153], but mutations in the parasite's cytochrome b gene appear to cause rapid resistance to this drug combination[154]. Other drugs, such as clindamycin, metronidazole and doxycycline have been tried to cure canine piroplasmosis with varying degrees of success. However, more controlled experimental studies are necessary to confirm the effectivity of these drugs[23].

6.2.2 Treatment in horses

Imidocarb dipropionate has been delisted for horses in Europe because of the lack of minimum residue limits for horsemeat, but can still be used under the cascade system. *B. caballi* infections are treated with IM administration of imidocarb dipropionate at 2.2 mg/kg for 2 treatments with a 24-48h interval. Sterilization of a piroplasm infection can be achieved by 4 IM injections of 4 mg/kg imidocarb dipropionate at 72h intervals[69]. Sterilization may be necessary in non-endemic regions or when a seropositive horse has to be moved from an endemic area to an EP-free region. Since *T. equi* is harder to treat, the high dose sterilizing protocol is immediately used when treating acute infections with this parasite. Clearance of the organism may also be achieved with this protocol. It should be noted that donkeys are especially susceptible to adverse reactions during treatment[56,69]. Other drugs have been used with variable success in the treatment of clinical signs, but cannot completely eliminate the parasite[60]. Diminazene aceturate therapy in horses is achieved by 2 treatments of 11 mg/kg 24h apart, and is only used when treating *B. caballi*. This therapy may result in elimination of the organism. Deep IM injections are recommended because swelling and necrosis at injection sites have been reported. Using multiple injection sites for the administration of smaller volumes of drug per site helps obviate these local reactions[60].

6.3 PREVENTION

Prophylaxis against tick borne diseases in general is based on vector prophylaxis with acaricides, repellants, chemoprophylaxis (targeted against the pathogen), vaccination and behavioral prophylaxis (reducing vector exposure by avoiding risk areas)[150]. Since adult *D. reticulatus* ticks are active throughout the year, a year-round tick prophylaxis is justified[78]. Transmission of the *Babesia* parasite occurs after a minimum of 2-3 days. If the feeding tick is removed earlier, chances of infection are minute. This means that frequent inspection of the skin and hair coat for ticks is important[114]. A variety of tick-removal tools are commercially available, such as tick-pliers and tick-twisters. For dogs, a variety of chemical prophylactic treatments in the shape of collars, spot-on formulations, sprays and chewable tablets are available on the European market. These topical ectoparasiticides either repel ticks and prevent attachment or kill ticks within 24-48h after application[22]. Recently, a new chewable tablet containing fluralaner (Bravecto®) proved to be effective in preventing the transmission of *B. canis* by infected *D. reticulatus* ticks over a 12-week period[155]. Another tablet containing afoxolaner (Nexgard®) proved to be effective over a 4-week period[156]. Acaricidal formulations applied by spray or powder may also be used to decrease tick burdens in the environment[22]. For horses, no such acaricidal products are registered in Europe. However, phoxim and pyrethroids are regularly used as a preventive treatment[157].

In some countries in Europe (such as France), a vaccine containing culture-derived SPA from a homologous *B. canis* strain is commercially available (Pirodog®). This vaccine does not prevent infection, but decreases the severity of clinical signs, parasitaemia and duration of clinical disease[22]. However, variable efficacy of the vaccine has been noted and attributed to strain variation[158]. Another vaccine (Nobivac® Piro), providing broader protection by containing SPA from heterologous origin of *B. canis* and *B. rossi*[22], has been licensed in the EU for some years, but is currently unavailable. No vaccines against EP are available for horses in Europe.

Experimental studies have suggested that a single dose of imidocarb dipropionate at a dose of 6 mg/kg protects dogs from infection for up to 8 weeks[159], and that doxycycline at 5 mg/kg per day reduces disease severity[160]. However, none of these prophylactic treatments has been consistently reliable in this regard[21,150]. In horses, a 3 mg/kg injection with imidocarb dipropionate has been reported to protect against *B. caballi* for 3-6 weeks[157,161].

CONCLUSION

It is safe to assume that the spread of piroplasmosis in Europe is correlated with the spread of *D. reticulatus*. However, the specific reasons for this spread are not so straightforward. A combination of climatological and human factors seems to be the most likely reason for this phenomenon. In these non-endemic regions, it is important to inform veterinarians that piroplasmosis is an increasing threat and should be included in the differential diagnosis when an animal shows signs of anemia.

A good epidemiological model to plot the changes in tick population distribution may help to predict these changes, so early preventive measures can be taken. Furthermore, especially dog owners should be educated about proper tick prevention. Since sterilization of the infection is hard to achieve and chronic carriers are common, more and more indigenous *D. reticulatus* populations will carry these parasites.

With the recent rise of PCR-technology, diagnosis of piroplasmosis could become more sensitive than the current commercially available diagnostic tests. This may improve the diagnosis of carrier animals, and thus more effective control measures can be taken. Finally, a proper vaccine against piroplasmosis may become commercially available in the future.

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