Résumé

Les maladies transmises par les tiques ont, partout dans le monde, un effet dévastateur aussi bien pour le bétail et les animaux de compagnie que pour les êtres humains. Les espèces de *Babesia* sont des protozoaires intraerythrocytiques transmis par une morsure de tique et qui parasitent un large éventail de mammifères domestiques et sauvages. En Europe, *Ixodes ricinus* est le principal vecteur de *Babesia* sp. et la voie de transmission la plus importante. D'autre part, *Rhipicephalus* (*Boophilus*) *microplus* est le vecteur le plus important d'un point de vue économique en Amérique Latine, Afrique et Australie. Les babésioses restent des maladies infectieuses parmi les plus communes chez les animaux sauvages et le bétail partout dans le monde et ont été récemment considérées comme des zoonoses potentiellement émergentes. En effet, depuis les années 50, deux espèces du genre *Babesia*, à savoir *B. divergens* en Europe et *B. microti* aux Etats-Unis, ont été associées à un nombre significatif de cas cliniques chez l'homme. La présence de ces espèces en Belgique a d'ailleurs pu être confirmée durant les études présentées dans cette thèse par l'utilisation de la réaction de polymérisation en chaîne (PCR) et du séquençage à partir des tiques récoltées sur différents hôtes vertébrés et dans l'environnement. Une augmentation du nombre d'infection humaine a été reportée depuis la dernière décennie. Certaines de ces atteintes ont été causées par des espèces nouvellement décrites telle que, *Babesia* sp. EU1. Celle-ci a pu être observée pour la première fois en Belgique ainsi que *Babesia capreoli*, espèce également inféodée aux cervidés. Les différentes études composant ce manuscrit ont pu déterminer un taux d'infestation des tiques par *Babesia* spp. compris entre 1,3 et 14,6% en fonction de l'hôte ou du lieu de récolte. Une séroprévalence de 14,3% a été observée chez des bovins pâturant en zone d'endémie tandis que des séroprévalences allant de 9 à 40% (en fonction de l'espèce de babésie considérée) ont été observées dans un groupe à risque chez l'homme (individus exposés à au moins une morsure de tique). Certaines co-infections ont pu être mises à jour, telles que celles par *Borrelia burgdorferi*, l'agent de la borréliose de Lyme, et *Anaplasma phagocytophilum*, l'agent de l'anaplasmose granulocytaire. L'étude sérologique portant sur la babésiose humaine est la première à utiliser les antigènes spécifiques des trois espèces de *Babesia* potentiellement zoonotiques en Europe. Ces antigènes ne semblent pas donner lieu à des réactions croisées. De plus, l'expansion géographique de certaines tiques vectrices de *Babesia* spp. a été étudiée en utilisant l'exemple de *Dermacentor reticulatus*, vecteur de la babésiose canine et équine, ainsi que *Rhipicephalus* (*Boophilus*) *microplus*, vecteur de la

babésiose bovine en milieu tropical. L'expansion de ces vecteurs pourrait jouer un rôle important dans l'épidémiologie de certaines babésioses.

Summary

Tick-borne diseases are devastating infections for livestock, companion animals and humans over most of the world. *Babesia* spp. are intraerythrocytic protozoan parasites that are primarily transmitted by a tick bite and include a number of species that are parasites of a broad range of domestic and wild mammalian hosts. In Europe, *Ixodes ricinus* is the most important vector and provides the main route of transmission. On the other hand, *Rhipicephalus* (*Boophilus*) *microplus* is the most economically important vector in South America, Africa and Australia. Babesiosis remains one of the most common infectious diseases of free-living animals and livestock worldwide and has recently been considered as a possible emerging tick-borne zoonosis. Indeed, since 1950, two species of *Babesia*, namely the cattle parasite *B. divergens* in Europe and the rodent parasite *B. microti* in the USA, have been associated with a significant number of clinical cases in humans. From experimental studies reported in this thesis evidence for these *Babesia* species in Belgium has been confirmed by Polymerase Chain Reaction (PCR) and sequencing performed on ticks collected from various vertebrate hosts and the environment. An increasing number of human infections were reported over the last decade, some of which being identified as caused by newly recognized *Babesia* species. One of these, *Babesia* sp. EU1, was described for the first time in Belgium as was *Babesia capreoli* a species also encountered in cervids. Other experimental work determined a *Babesia* infection rate in ticks at between 1.3 and 14.6% according to the host and place of collect. A seroprevalence of 14.3% was recorded in cattle grazing in an endemic area of Belgium. Seroprevalences ranging between 9 and 40% (depending on the *Babesia* species detected) were found in humans exposed to at least one tick bite. Co-infections of *Babesia* with *Borrelia burgdorferi*, the agent of Lyme Borreliosis and *Anaplasma phagocytophilum*, the agent of granulocytic anaplasmosis were detected. The serological survey on human babesiosis performed in this work is the first to use specific antigens for all three potentially zoonotic species in Europe. These antigens were apparently not responsible for cross reactions. Expansion of some *Babesia* tick vectors was studied using the example of *Dermacentor reticulatus*, a vector of canine and equine babesiosis, and

Rhipicephalus (*Boophilus*) *microplus*, a vector of bovine babesiosis in tropical regions. Expansion of these vectors could play an important role on the epidemiology of babesiosis.

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°C: degree Celsius
%: Percentage
bp: base pair
BGA: Bovine granulocytic Anaplasmosis
CA1-6: California 1-6
CBC: Complete Blood Count
CI: Confidence interval
DAPI: 4', 6-diamidino-2-phenylindole
DNA: Deoxyribonucleic Acid
EU1: Europe 1
ELISA: Enzyme-linked immunosorbent assay
HGA: Human Granulocytic Anaplasmosis
HIV: Human Immunodeficiency Virus
IFAT: Immunofluorescent antibody test
Ig M/G: Immunoglobulin M/G
INFγ: Interferon gamma
IPM: Integrated Pest Management
ITS: Internal Transcribed Spacer
kg: kilogram
KO1: Korea 1
LAMP: Loop-mediated isothermal Amplification
mg: milligram
ml: milliliter
µl: microliter
µm: micrometer
NO: Nitric oxide
PCR: Polymerase Chain Reaction
RFLP: Restriction Fragment Lenght Polymorphism
rpm: revolutions per minute
s: second
sp./spp.: species
rRNA: ribosomal Ribonucleic Acid
SPA: Soluble Parasite Antigens
TBE: Tick-borne Encephalitis
TBD: Tick-borne Disease
TBF: Tick-borne Fever
TNFα: Tumor necrosis factor alpha
UK: United Kingdom
USA: United States of America
WA1-3: Washington 1-3

I. Introduction

Tick-borne diseases are devastating infections for livestock, companion animals and humans over most of the world. The diseases cause high economic losses to the livestock industry, are an animal health and welfare issue and can be a significant threat to public health.

The genus *Babesia* belongs to the phylum Apicomplexa and includes a number of species that are parasites of a broad range of domestic and wild mammalian hosts. *Babesia* parasites were first identified in 1888 by Victor Babes, a Romanian scientist, who described a Plasmodium-like parasite as a cause of febrile haemoglobinuria in cattle (Babes, 1888).
Babesia spp. are intraerythrocytic protozoan parasites that are primarily transmitted by a tick bite, but can also be transmitted via blood transfusion. Different tick vectors can transmit these diverse species. In Northern Europe, *Ixodes ricinus* is the most important vector and provides the main route of transmission. On the other hand, *Rhipicephalus* (*Boophilus*) *microplus* is the most economically important vector in South America, Africa and Australia where losses from associated tick-borne pathogens have been estimated in hundreds of millions of US dollars to cattle producers. Babesiosis, also named "red water fever" remains one of the most common infectious diseases of free-living animals and livestock worldwide and has recently been considered as a possible emerging tick-borne zoonosis (Homer et al., 2000; Foppa et al., 2002; Hunfeld and Brade, 2004). Indeed, since 1950, two species of *Babesia*, namely the cattle parasite *B. divergens* in Europe and the rodent parasite *B. microti* in the USA, have been associated with a significant number of clinical cases in humans. An increasing number of human infections have been reported over the last decade, some of which have been identified as caused by newly recognized *Babesia* species (Hunfeld and Brade, 2004).

The following general introduction to this thesis will summarize the current knowledge about *Babesia* spp., their tick vectors and associated diseases in vertebrate hosts.

A. Life cycle

The life cycle of *Babesia* species is shared between a phase in an invertebrate host tick and a phase within a vertebrate mammalian host, changing their morphology, location and their ploidy as they form different stages of the cycle.

Invertebrate host

Ticks become infected by the ingestion of uni-nucleated gametocytes during their blood meal from the vertebrate host. These gametocytes develop into gametes, described as ray bodies (Strahlenkörper), elongated cells with spiny extensions, inside the tick gut. Ray bodies cannot be differentiated with respect to sexual status by light microscopy. In electron microscopy, cells with a dense cytoplasm have been distinguished from other forms that display clear cytoplasm, leading to hypothesis of a sexual cycle (gametogony). Following fusion of two ray bodies (gametes), a spherical zygote is formed, which then differentiates to produce mobile polyploid kinetes of 7-8 µm. These kinetes enter the tick haemolymph and are disseminated throughout various tissues, including the musculature, Malpighian tubules, epidermis, and ovaries. One or two cycles of asexual reproduction follow, resulting in high numbers of kinetes. Developing tick eggs may be invaded, giving rise to kinete infected larvae (transovarial transmission). Once the next stage in the tick life cycle starts to feed on a new host, kinetes that have entered the cells of the tick salivary glands develop into numerous uni-nuclear sporozoites (sporogony). Inoculation of the infective sporozoites occurs during the latter half of the blood meal (transstadial transmission) (Figure 1).

Vertebrate host

Vertebrate hosts become infected during the feeding of infected ticks on that host. *Babesia* spp. invade erythrocytes directly; after attachment of the sporozoite to the host cell and orientation of the apical complex towards the erythrocyte surface, secretory products released by the rhoptries cause the invagination of the erythrocyte membrane. However, for at least

two species, *Babesia* (*Theileria*) *microti and Babesia* (*Theileria*) *equi*, sporozoites invade the lymphocytes of their hosts. This characteristic has prompted some authors to state that these two species should be placed in the genus *Theileria*. Following invasion, the vacuole membrane disintegrates and the parasite is in direct contact with the erythrocyte cytoplasm. At this stage the parasite is described as a trophozoite that divides asexually by binary fission to form two haploid merozoites, leading to the characteristic appearance of paired parasite within the infected erythrocyte, which is often used as the basis of diagnostic identification. In some species, four parasites are often formed within the erythrocyte and appear as a tetrad or "Maltese cross". Merozoites are released from the red cell as it lyses and rapidly invade new ones. The vast majority of merozoites continue to multiply asexually, while a small proportion turn into nondividing spherical gametocytes, which remain inside erythrocytes until they are taken up by ticks during feeding (Mehlhorn and Shein, 1984) (Figure 1).

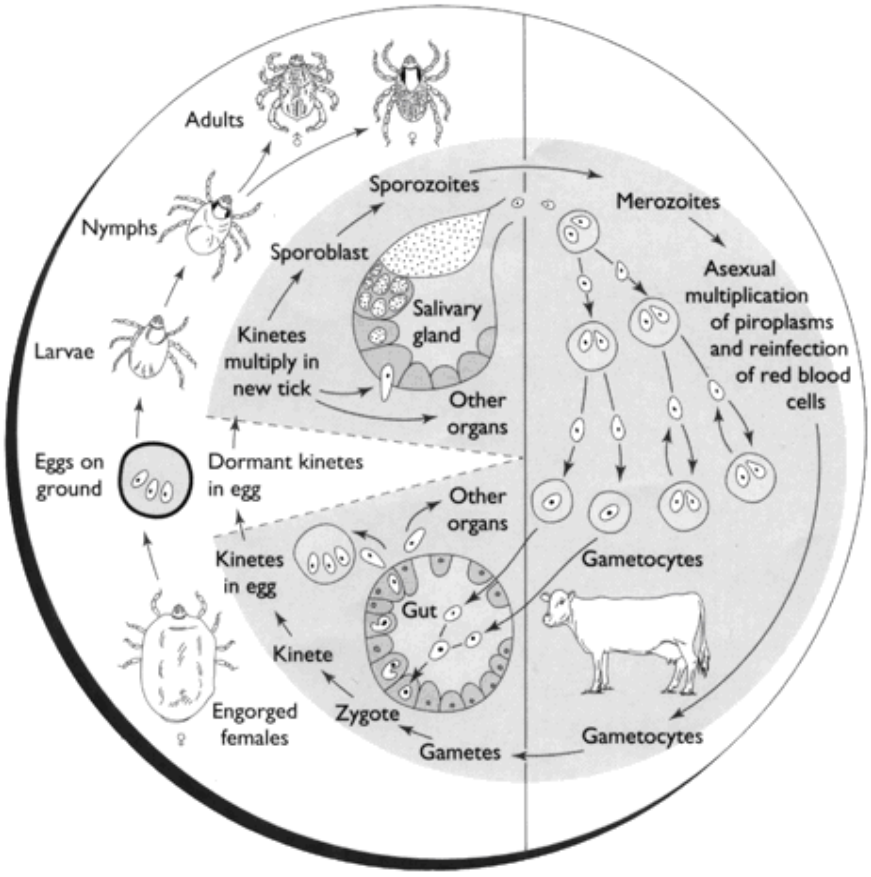

Figure 1: Life cycle of *Babesia* spp. Adapted from Mehlhorn and Schein (1984).

B. Morphology

Location, size and shape of the intraerythrocytic stages are characteristic of *Babesia* species and are used for diagnostic purposes. *Babesia* spp. occur exclusively in erythrocytes (except for *B.* (*T.*) *microti and T.* (*B.*) *equi*), are in direct contact with the cytoplasm of the red blood cells and are devoid of haemozoin pigment, unlike *Plasmodium* spp. The multiplication that occurs in erythrocytes by asynchronous binary fission, results in a wide range of morphological forms. All divisional stages and associated forms can be observed in the same blood smear from a parasitized host (Figure 3).

Light microscopy results in identification of a range of shapes. Ring or annular stages are round or oval and have a darker stained nucleus around the periphery; pear-shaped or pyriform trophozoites occur singly or in pairs assembled at their pointed extremities to form a sharp or obtuse angle, or may form the "Maltese cross" tetrad. Irregular forms can also be observed with a peripheral nucleus and vacuolar cytoplasm (Figure 2-9).

Morphology varies according to *Babesia* species described as large or small parasites. Small *Babesia* spp. measure 1.5 to 2 µm long, *B. bovis* and *B.* (*T.*) *microti* for example, and large *Babesia* spp. such as *B. canis* and *B. bigemina*, measure 2.5 to 3 µm long (Mehlhorn and Shein, 1984). Morphology can also differ with respect to *Babesia* parasites found in different host species. Thus, for the same *Babesia* species, the position inside the erythrocyte and morphological details can change according to the host species infected. Morphological variability has even been observed for *Babesia* parasites infecting different individuals of the same species or following concurrent infections. Moreover, shape polymorphism can occur according to the "*in vivo* or *in vitro*" status of infected red blood cells. The specific causes of these morphological and positional changes are not yet understood (Zintl et al., 2003).

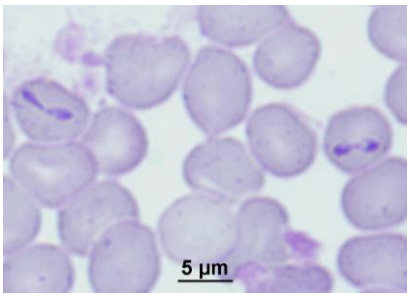

Figure 2: *Babesia divergens* on a Diff-Quick coloration smear (University of Liège-François Smeets).

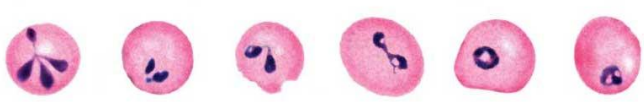

Figure 3: *Babesia* sp. EU1 on a Giemsa-stained smear (Herwaldt et al., 2003). On the left side, a tetrad or "Maltese cross", in the middle pairs and on the right side, ring forms.

Figure 4: *Babesia bovis* (www.dpi.qld.gov.au/4790_5838.htm).

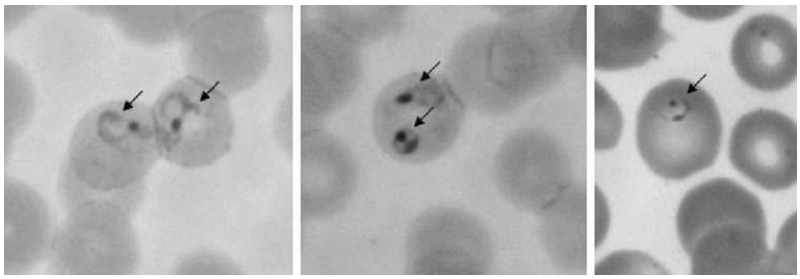

Figure 5: *Babesia* (*Theileria*) *microti* in a human patient in Europe (Hildebrandt et al., 2007).

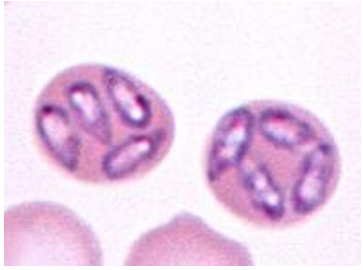

Figure 6: *Babesia canis* (ahdc.vet.cornell.edu/.../rbcmorph/b-canis.htm).

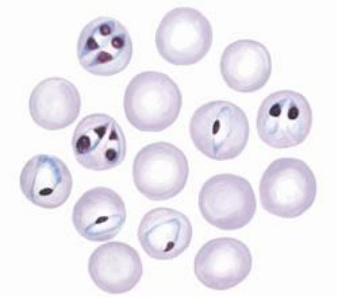

Figure 7: *Babesia bigemina* (www.dpi.qld.gov.au/4790_5838.htm).

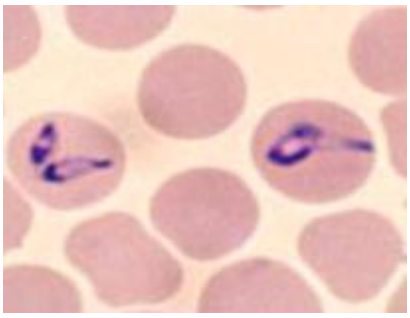

Figure 8: *Babesia caballi* (Naoaki Yokoyama).
(http://fullmal.hgc.jp/bb/icons/bovis_and_caballi.jpg)

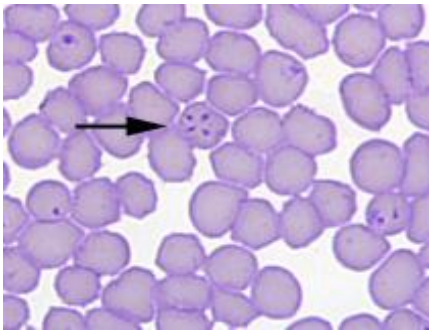

Figure 9: *Theileria* (*Babesia*) *equi* (Parasitologia-Universitat Autonoma de Barcelona).

C. Pathogenicity

Mechanisms able to produce pathological effects have to be elucidated in order to understand the origin of subsequent symptoms and lesions. The first pathological episode in the vertebrate host caused by *Babesia* infection is intravascular haemolysis due to merozoites exiting the erythrocyte, resulting in normocytic haemolytic anaemia (Furlanello et al., 2005;

Gohil et al., 2010) and consequently, in acute infections, hyperbilirubinemia (unconjugated bilirubin), haemoglobinuria and jaundice. Subsequent anoxia and toxic effects can cause organ failure and death. Thrombocytopenia is a common manifestation of all tick-borne diseases. However in many of the tick-borne diseases it is poorly understood. Quantitative changes in platelet counts associated with *Babesia* infection may result from hypersplenism, as well as immune-mediated platelet destruction. The spleen is usually severely affected in cases of babesiosis, becoming congested and enlarging to several times its original size. Enlargement of the spleen results in increased platelet sequestration and destruction by splenic macrophages. This reduces the circulating number of thrombocytes. Autoimmunity is a phenomenon observed in many tick-borne diseases, and may contribute to thrombocytopenia in babesiosis infection. Autoreactive antibodies that bind to platelets shorten their life span, due to the clearance of antibody-coated platelets by the reticuloendothelial system (Orinda et al., 1994; Pantanowitz, 2003).

Parasite antigens can also become fixed to the membrane of infected erythrocytes and promote phagocytosis by macrophages. Immune complexes can be produced and increase erythrocyte permeability or cause glomerulonephritis and associated pathology. Symptoms such as fever and myalgia could be due to an excessive production of pro-inflammatory cytokines by the host, as described for malaria (Krause et al., 2007; Gray et al., 2010).

The pathogenicity of *B. bovis* seems to be more complex than described for *B. divergens* or *B. bigemina*, as it is associated with intra vascular coagulation and vasoactive shock. These symptoms can occur at low parasitaemias (<1%). The syndrome of intravascular aggregation in the cerebral cortex is pathognomonic of infection by *B. bovis*. Sequestration of infected erythrocytes in cerebral and pulmonary capillaries results in a low parasitaemia in the peripherical circulation but potentially high in cerebral capillaries. This phenomenon can allow escape from the host immune system by preventing passage through the spleen and facilitates persistent infection (Krause et al., 2007).

D. Clinical symptoms and lesions

In general, clinical signs of babesiosis range from subclinical to death. The severity of infection (mild, severe, fatal) depends on the number of infected ticks feeding on the animal but is also influenced by the virulence of the strain and the immune status of the host (Zintl et al., 2003).

Clinical signs of disease begin with anorexia, weakness and depression combined with high fever (up to 41 °C) (Figure 10), followed by anaemia (Figure 11) and diarrhoea with spasms of the anal sphincter. As the infection advances, dehydration becomes severe and diarrhoea is replaced by constipation (Figure 12). Icterus may develop as a result of haemolysis. Brain anoxia resulting from severe anaemia may cause behavioural changes (Gray and Murphy, 1985). In addition nervous signs can be found in cases of *B. bovis* infection due to sludging of infected red blood cells in the capillaries of the host's brain. Haemoglobinuria is a conspicuous sign of babesiosis (frequently the first clinical symptom seen by the farmer) and occurs during the peak of haemolysis (Figure 13). Death is generally attributed to cardiac failure or hepatic and renal insufficiency (Collins et al., 1970). Speed of diagnosis is very important as it influences the recovery rate following treatment.

Figure 10: Weakness and depression in cattle afflicted by babesiosis (osp.mans.edu.eg/.../bovine%20babesios.htm).

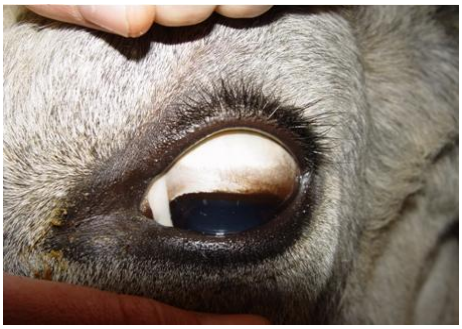

Figure 11: Cattle with pale mucosae probably due to anaemia (Pf. Claerebout-University of Ghent).

Figure 12: Cattle showing constipation (Pf. Sahibi and Rhalem-IAV Hassan II Morocco).

Figure 13: Haemoglobinuria associated with acute disease (Pf. Losson, Dr. Mignon-University of Liège).

The clinical symptoms of babesiosis in wild animals appear to be the same as their domestic counterparts but are less well documented (Penzhorn, 2006; Kik et al., 2011).

Characteristic lesions of babesiosis are marked jaundice (Figure 14), enlarged and darkened liver and kidneys (Figure 15), and a swollen spleen with a soft pulpy consistency (Figure 16). The bladder is distended and full of dark urine (Figure 17). Ecchymotic haemorrhages may be present under the epicardium and the endocardium. The pericardial sac may contain a large

quantity of blood-stained fluid (Collins et al., 1970; Gray and Murphy, 1985). The cerebral form of *B. bovis* shows pink haemorrhages and formation of thrombi and emboli in brain capillaries (Figure 18).

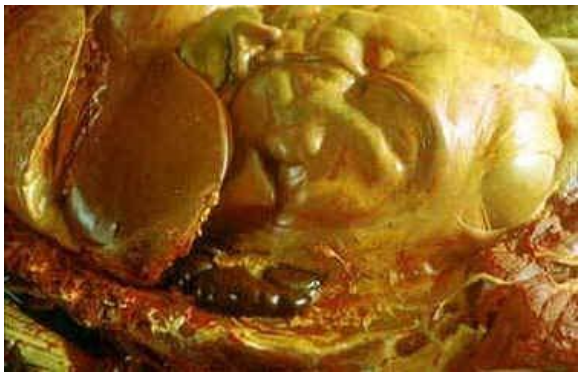

Figure 14: General lesions: the entire carcass may be icteric and/or pale. (University of Georgia, http://www.vet.uga.edu/vpp/archives/NSEP/babesia/ENG/necropsy_findings.htm)

Figure 15: The liver can be enlarged and associated with icterus. (University of Georgia, http://www.vet.uga.edu/vpp/archives/NSEP/babesia/ENG/necropsy_findings.htm)

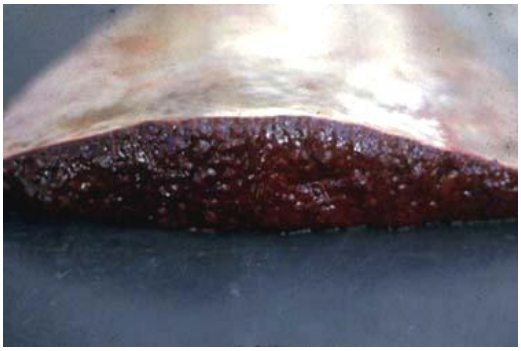

Figure 16: The spleen is enlarged and soft. (University of Georgia, http://www.vet.uga.edu/vpp/archives/NSEP/babesia/ENG/necropsy_findings.htm)

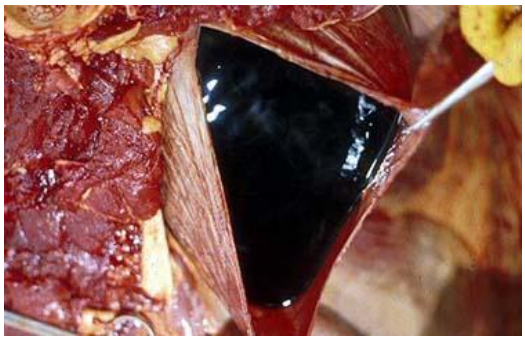

Figure 17: The urinary bladder is distended and full of dark urine. (University of Georgia, http://www.vet.uga.edu/vpp/archives/NSEP/babesia/ENG/necropsy_findings.htm)

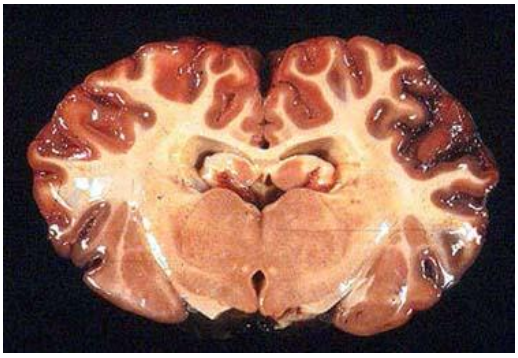

Figure 18: The cerebral form of babesiosis caused by *B. bovis* is characterized by formation of thrombi and emboli in brain capillaries. (University of Georgia, http://www.vet.uga.edu/vpp/archives/NSEP/babesia/ENG/necropsy_findings.htm)

E. Immunology

Immunity to *Babesia* infections is conferred by both the innate and acquired immune response and is influenced by age and the presence of a spleen. Young calves can be protected by colostral immunity during the first 2 or 3 months, depending on the immunological status of their dams. Moreover, a strong innate immunity occurs in calves of 3-9 months of age and can be present in calves without colostral protection (Goff et al., 2010). This "inverse age resistance" phenomenon makes young calves and foals less likely to respond with clinical symptoms (Zintl et al., 2003) if infected within the first year. This is not the case for puppies,

kids and lambs affected by *Babesia* spp. (Zintl et al., 2005). Innate resistance seems to be antibody-independent and is thought to depend primarily on an early Type I response involving IFN-γ and TNF-α, which activate macrophages to release nitric oxide (NO). NO seems to be a short-lived babesiacidal molecule with its effect restricted to lymphoid organs such as the spleen. Excessive NO production, however, could be responsible for some clinical symptoms (Krause et al., 2007; Goff et al., 2010). The spleen plays an important role in both specific and non-specific immunity, as it functions to remove altered or infected red blood cells from the circulation. Splenectomy increases the susceptibility to *Babesia* infection and results in a loss of the natural immune resistance of young calves. Additional mechanisms of innate resistance that have been proposed are the inhibitory effect of foetal haemoglobin and the differential susceptibility of the reticulocytes in young calves (Zintl et al., 2005). Specific immune mechanisms include both cellular and humoral components. Monocytes and lymphocytes are thought to be the main agents of cell-mediated acquired immunity. These monocytes are thought to engage in antibody-dependent cell mediated cytotoxicity. In vitro experiments have also demonstrated a role for $CD4^+$ helper T cells. These T cells produce cytokines, of which IFN γ seems to be of major importance, which activate phagocytic cells and enhance antibody production by B cells. The exact role of antibodies is poorly understood; maybe they inhibit merozoite invasion of erythrocytes or stimulate phagocytosis of the infected red blood cell such as opsonins rather that having a direct effect on the parasite (Zintl et al., 2003; Bock et al., 2004). After recovery a long-lasting immunity occurs, but not indefinitely.

F. Diagnosis

Babesiosis is generally indicated by the clinical symptoms and usually confirmed by detection of pyriform organisms in Giemsa-stained blood smears together with haemolytic anaemia and an elevated reticulocyte count. Morphology is not useful to identify closely related *Babesia* parasites, especially since the appearance of a particular species may vary in different hosts (Gray, 2006). Serological tests [Immunofluorescent antibody test (IFAT), Complement

fixation assay or Enzyme-linked immunosorbent assay (ELISA)] can also confirm a primary diagnosis or be used for epidemiological surveys. IFAT is the most popular serological technique and can clearly differentiates the majority of *Babesia* spp. Laboratory animal inoculation, hamster or gerbils (*Meriones unguiculatus*), and *in vitro* culture are also used for diagnosis purposes but only in the research laboratory. Such tests are not useful in emergency situations and not suitable for all *Babesia* spp. such as for *Babesia* sp. EU1, as it does not proliferate in gerbils (Herwaldt et al., 2003; Häselbarth et al., 2007). A number of molecular tests have been developed and implemented and are mainly based on analysis of the 18S rRNA gene or β-tubulin gene. These tests are more sensitive with comparable specificity than more traditional parasitology based assays and allow detection of low parasitaemia. Molecular based tests are useful for diagnosis, detection in the tick vector, and also for differential diagnosis against other tick-borne diseases such as anaplasmosis and Lyme borreliosis.

In humans, diagnosis of babesiosis is sometimes difficult especially during the initial phase of the infection because of the relatively non specific clinical signs, although in cases of acute babesiosis, haemoglobinuria is easily detected. Diagnosis can be further complicated by the long persistence of subclinical infections with *B.* (*T.*) *microti* (Krause et al., 1998) and may underlie other vector-borne diseases (Hunfeld and Brade, 2004), particularly Lyme borreliosis and malaria (Hunfeld et al., 2008). Morphologically, the ring form can be confused with *Plasmodium* but the absence of haemozoin pigment in *Babesia* sp. allows differentiation (Gray et al., 2010). Parasites are often not visualized early in the course of infection due to a low level of parasitaemia, particularly in an immuno-competent host (Hunfeld and Brade, 2004). Screening tests such as complete blood count (CBC), platelet count or assaying liver enzymes could be performed before requesting a specific test. Indeed, thrombocytopenia and elevated levels of serum liver enzymes are present in about half of babesiosis patients (Vannier and Krause, 2009). Serological tests are most often used for epidemiological survey, particularly in investigations of blood transfusion-related babesiosis. Various assays have been developed for the screening of IgM and/or IgG; the most commonly used method being the IFAT. ELISA tests for *B.* (*T.*) *microti* can be used to confirm IFAT results, but are not currently available commercially and not sufficiently standardized for routine application (Hunfeld et al., 2008). Molecular based tests are also used for diagnosis and are useful for monitoring the efficiency of anti-babesial therapy and for differential diagnosis against other vector-borne diseases.

Diagnostic tools	Sensitivity/Specificit	Feasibility	Period of time	Cost
Microscopic examination (blood smear)	relatively low	easy (possible in the field)	course of infection	cheap
Blood analysis (CBC…)	relatively low	easy	course of infection	cheap
Animal inoculation	relatively low	difficult (only in research laboratory)	course of infection	cheap
Serology (IFAT, ELISA, Complement assay, immuno-blot)	relatively high	time consuming	weeks after infection (useful for epidemiological survey)	relatively expensive
Molecular tools (PCR, LAMP…)	relatively high	time consuming	course of infection (suitable for tick vector analysis)	relatively expensive

Table 1: Characteristics of different diagnostic tools for *Babesia* spp.

G. Treatment and control

Treatment

For many years, three drugs: quinuronium sulfate (Zothelone®, Babesan®), amicarbalide isethionate (Diampron) and diminazene aceturate (Berenil®), have been extensively used in the tropics as both babesicides and trypanocides, and were available in most European countries for the treatment of bovine babesiosis. Later, imidocarb diproprionate (Imizol®, Carbesia®) was introduced, and rapidly became the product of first choice because, in

addition to its therapeutic efficacy, it also proved to be an effective prophylactic product. Today many of the most effective drugs against *B. divergens* have been withdrawn due to safety and residue problems or for marketing reasons, and imidocarb diproprionate is the only product on the market in most of Europe (Zintl et al., 2003), and under the cascade system in Belgium. Unfortunately, withdrawal periods for imidocarb can be long, especially for meat consumption (28 days), and adverse effects may occur following administration of this drug at high doses. To aid recovery, supportive treatments are often administered, such as iron, vitamins, purgatives and fluid replacements; blood transfusion is highly recommended for animals with acute anaemic anoxia. Treatment with non-steroidal anti-inflammatories can be used to reduce the negative effect of excessive NO production (Zintl et al., 2005).

In humans, several drugs have been evaluated *in vitro* but their use is associated with significant side effects and treatment failure (Hunfeld et al., 2008). Imidocarb is highly effective *in vitro* and has been used successfully to treat patients infected with *B. divergens* (Zintl et al., 2003) but is not approved for general use in humans. To date, the main treatment is a combination of quinine and clindamycin over 7 to 10 days, but the side effects of quinine demonstrate a requirement to look for novel drugs. A combination of atovaquone (Mepron®) and azithromycin (Zithromax®) as an association atovaquone-proguanil (Malarone®) (Vyas et al., 2007) has been proposed for treatment of *B.* (*T.*) *microti* infection, as this therapy proved to be as effective as the standard quinine/clindamycin combination, but with fewer side effects and consequently safer if the drug must be used several times which is often the case as relapsing infections are possible. Most of the infections due to *B.* (*T.*) *microti* resolve on their own, chemotherapy only being indicated for moderate to severe cases (Hunfeld et al., 2008). However, clinical infections due to *B. divergens* are often diagnosed as an emergency. Treatment is then needed immediately and is often combined with exchange of blood via transfusion. In HIV and other immuno-suppressed patients the efficacy of the drugs are significantly reduced (Gray et al., 2010) and higher dosage and prolonged administration are needed (Froberg et al., 2004; Krause et al., 2008). Differences in susceptibility of other *Babesia* species to the drugs commonly used to treat human babesiosis have not been recorded.

Control

Prevention of *Babesia* spp. infections begins by avoiding tick bites and by tick control. The best way to avoid an infection is to know where, when and how it can be acquired. This is why public information is a pillar in preventive measures schemes. Each tick species has its own peak of activity periods and specific environmental requirements; for some species, spatial and temporal patterns of risk exposure can be defined. This information could be used to develop strategy to minimize exposure and may be used for diagnosis purposes (Piesman and Eisen, 2008).

Preventive action can be achieved by daily checks and prompt tick removal. In humans, personal protection can be achieved by wearing appropriate clothes and the use of arthropod repellents. The use of acaricides is a common measure to control tick exposure on animals but can also be applied to vegetation. Sometimes these products are not correctly used on animals, allowing development of strong acaricide resistance. In some tropical regions, the emergence of resistance to several chemical classes such as arsenicals, organophosphates, organochlorines, formamidins, macrocyclic lactones or pyrethroids have caused severe economic consequences for cattle producers (George et al., 2004). The use of acaricides on vegetation can have a negative impact by causing toxicity and ecological damage, even if successful decrease in the public health risk of tick-borne agents has been shown (Piesman and Eisen, 2008). However new acaricide delivery systems have been developed based on a host-targeted approach. Thus the 4-poster device for deer (Figure 19) and cotton ball for rodents (Piesman and Eisen, 2008) are designed to control ticks on wild animals by targeting the limited amount of acaricide deployed in the environment towards the most important mammalian hosts.

Figure 19: 4-poster device for deer. The design of the device encourages the deer to tilt its head toward the application rollers, ensuring that acaricide is transferred to its head, ears and neck (USDA ARS/Wayne Ryan).

Vegetation management (bush removal, burning) is another solution used to avoid extensive use of acaricide and some biological agents such as entomopathogenic fungi have also been investigated for tick control (Hornbostel et al., 2005). These fungi are potential agents for biological control since all tick stages have been found naturally infected by several species of these organisms such as *Metarhizium anisopliae* and *Beauveria bassiana* and have shown efficacy against various stages of many tick species. Under laboratory conditions, these acaripathogenic fungi can cause high mortality in all developmental stages of several tick species, and also reduce oviposition of infected engorged females. However, their efficacy declines considerably under field conditions since fungal action is affected by many negative biotic (isolates, virulence, tick immune response) and environmental factors such as temperature, humidity, solar radiation, rainfall (Camargo et al., 2012; Fernandes et al., 2012). Limitation of deer populations by fencing or hunting was also used to control tick populations (Rand et al., 2004), but with mixed success. Anti-tick vaccines, such as the commercial tick vaccine for cattle based on the *Rhipicephalus* (*Boophilus*) *microplus* Bm86 gut antigen (TickGard®, Gavac®), have been developed as an alternative to the acaricide as they potentially offer a cost-effective and environmentally friendly solution (de la Fuente et al., 2007). Such vaccines, however, may require yearly application to large reservoir populations of wild hosts in certain epidemiological situations and use a single antigen, which seems does not appear to be sufficient to provide optimal efficacy.

The impact of host genotype on host resistance to ticks and tick-borne diseases is complex but very important. For example, *Bos indicus* or crossbreed cattle show increased survival rates against babesiosis (*B. bovis* and *B. bigemina*) compared to *Bos taurus* animals (Bock et al., 1997). Furthermore differences in the ability of cattle to become resistant to ticks, whether *Bos indicus* or *Bos taurus* or within *Bos taurus* breeds, has long been recognized, as has the finding that the ability to acquire resistance is heritable (Utech and Wharton, 1982).

Unfortunately, there is no single, ideal solution for the control of ticks and integrated pest management (IPM) schemes involving the application of two or more technologies to control pest populations which negatively affect a host species (Bram, 1994) are likely to be required. The ultimate aim of such strategies is to achieve parasite control in a more sustainable, environmentally friendly and cost-effective method than is achievable with a single technology (Willadsen, 2006). It is debatable whether traditional and current control methods, primarily based on drugs will be able to meet these goals.

In addition to tick control, drugs can be used to prevent clinical babesiosis. Imidocarb, as already mentioned, can be employed as a prophylactic treatment using twice the treatment dose. This approach is most effective in an environment where the host is guaranteed to contract babesiosis, as clinical disease is prevented while immunity to further infection is promoted. Long-acting oxytetracyclin can also be used for prevention with 4 injections at 20 mg/kg weekly, but not for therapeutic purpose. No drugs are available for this approach in humans and owing to the scarcity of the disease, it is most unlikely that vaccines will be developed as the preferred strategy for control of babesiosis in humans (Gray et al., 2010).

Different types of vaccine have been implemented against cattle babesiosis including live or dead whole parasites or preparations of isolated antigens. Attenuated live vaccines have proven their efficacy particularly in young cattle when they still have natural resistance. Live vaccines have several drawbacks such as the potential transmission of other pathogens, the sensitization to bovine red blood cell antigens when vaccines are produced from splenectomised calves, a short shelf life and the requirement of an effective cold chain. In addition, even with a controlled attenuation, vaccine strains can still cause babesiosis. In this case, virulence can be controlled by the use of imidocarb a few days before vaccination (Zintl et al., 2003). Recombinant vaccines or exoantigens based vaccines have been tested with variable success. None are widely used against bovine babesiosis but commercial vaccines against canine babesiosis are available in some countries (Pirodog®, Nobivac Piro®). Pirodog® vaccine contains soluble parasite antigens (SPA) from *B. canis* and Nobivac Piro®

contains in addition SPA from *B. rossi*. Vaccines based on dead whole parasites have been tested without conclusive results.

H. Epidemiology

To understand the epidemiology of babesiosis, different relationships between the parasites and their hosts have to be taken into account. Protection conferred by the vertebrate host immune system in response to *Babesia* spp. will depend on age, breed, and immune status. The inverse age resistance that protects young calves against clinical disease has a strong influence on promoting enzootic stability. In this situation, endemic infection levels are in balance with herd immunity, as animals infected when calves become immune adults, with immunity constantly boosted on subsequent rechallenge. Clinical disease is rare in these endemic areas (Zintl et al., 2005). In dogs, like cattle, some breeds (Beagle, Fox terrier, Porcelain, Dachshund, mongrels) show a higher resistance to *Babesia* spp. infection than others (Spaniel, Cocker spaniel, Griffon, Yorkshire terrier, Doberman, Pekinese) (Martinod et al., 1986). The different level of breed resistance probably has a genetic basis (Chauvin et al., 2009). In the face of an effective immune response, *Babesia* spp. have developed stratagems to avoid or limit total clearance by the host immune system. Antigenic variation of surface proteins responsible for cytoadherence and diversity of antigenic epitopes recognized by the immune response prolong the persistence of parasite in the vertebrate host and increase the chance of transmission to the tick vector. Persistence of *Babesia* spp. in the tick vector and mechanisms that transmit infection also influence the epidemiology of babesisosis and can facilitate long-lasting persistence of the parasite in an endemic region, even in the absence of the specific vertebrate host. Moreover, a wide range of species acting as the host for the tick vector can contribute to the development and adaptation of *Babesia* spp. to new mammalian host species (Chauvin et al., 2009). Geographical distribution of *Babesia* spp. is highly associated with tick distribution, tick population dynamics and the relationship of the tick vector with its vertebrate hosts and the environment. Climate and environmental change, such

as altered land use for human recreational activities, can contribute to the emergence or an increase in tick-borne diseases.

I. The main vector ticks of *Babesia* spp.

Ticks are parasites placed in the subclass of Acari and the order Ixodida. Throughout history, ticks have been condemned for their parasitic activities. Aristotle in Historia Animalum considered that ticks are "disgusting parasitic animals…"; they spend their lives living in dirt, feeding on the blood of animals or humans and provide a source of infections. However, study of ticks has revealed a fascinating biology with a tremendous potential for environmental adaptation and survival. Theobald Smith and Frederick Kilbourne first demonstrated (1889–1893) that ticks were important vectors of disease following transmission of *Babesia bigemina* by *Rhipicephalus* (*Boophilus*) *annulatus* in cattle (Assadian and Stanek, 2002). This work brought attention to ticks as sources of potential significant losses to livestock production through transmission of disease together with direct damage caused by feeding activity.

Babesia spp. are transmitted to their vertebrate hosts by ticks, within which parasite sexual reproduction occurs. Specificity for the tick host is generally less strict than for the vertebrate host. The specific vectors of many species of *Babesia* are still to be discovered, however, *Babesia* spp. of veterinary importance and zoonotic potential are known to be transmitted by ixodid ticks listed in Table 2 (Hunfeld et al., 2002). Transmission of *Babesia* spp. to vertebrate host can be present depending on tick stages (larvae, nymph or adult). This is mostly related to mechanism of parasite transmission (transstadial or/and transovarial), vertebrate host specificity of these different tick stages and *Babesia* species rather than a difference on vector competence between the different tick stages.

Tick Species	***Babesia* Species**	**Tick stages**	**Other Main Pathogens**
R.(*Boophilus*) *microplus*	*B. bovis, B. bigemina*	all	*Anaplasma marginale*
R.(*Boophilus*) *decoloratus*	*B. bigemina*	all	*Anaplasma marginale, Borrelia theileri*
R.(*Boophilus*) *annulatus*	*B. bovis, B. bigemina*	all	*Anaplasma marginale*
R.(*Boophilus*) *geigy*	*Poorly known, B. bovis?*	all	
R. sanguineus	*B. canis vogeli, B. gibsoni*	all	*Ehrlichia canis, Rickettsia conorii, Theileria equi*
Haemaphysalis punctata	*B. major, B. motasi*	mainly adult	*Theileria ovis, Theileria buffeli, Anaplasma marginale, Anaplasma centrale, Coxiella burnetii, Francisella tularensis, TBE*
Haemaphysalis elliptica	*B. canis rossi*	all	
Dermacentor reticulatus	*B. canis canis, B. caballi*	mainly adult	*Theileria equi*, Tick-borne encephalitis virus (TBE), *Rickettsia conorii*, *Rickettsia slovaca*, *Francisella tularensis* and *Coxiella burnetii*
Ixodes hexagonus	*B.* (*T.*) *microti*	nymph, adult	*Anaplasma phagocytophilum, Borrelia burgdorferi*, TBE
Ixodes ricinus	*B. divergens, Babesia* sp. EU1, *B.* (*T.*) *microti* in Europe	mainly adult	*Anaplasma phagocytophilum, Borrelia* spp., *Rickettsia* spp.
Ixodes scapularis	*B.* (*T.*) *microti* in the USA, *B. odocoilei*	nymph, adult	*Borrelia burgdorferi, Anaplasma marginale, Francisella tularensis*
Ixodes persulcatus	*B.* (*T.*) *microti, Babesia* sp. EU1, *B. divergens, B. ovis*	nymph, adult	*Borrelia burgorferi*, TBE, *Anaplasma phagocytophilum*
Hyalomma spp.	*B. caballi*	all	*Theileria equi*

Table 2: Tick species that transmit *Babesia* spp. and other main pathogens and tick stages associated with transmission of *Babesia* spp.

1. Rhipicephalus (*Boophilus*) spp.

The genus *Boophilus* has recently been reclassified as a subgenus of *Rhipicephalus* and is composed by 5 different species: *R.* (*Boophilus*) *decoloratus, R.* (*Boophilus*) *annulatus, R.* (*Boophilus*) *geigyi, R.* (*Boophilus*) *microplus* and *R.* (*Boophilus*) *kohlsi*. From an economical point of view, Boophilids are some of the most important tick species in the world (Estrada-Peña et al., 2006).

Rhipicephalus (*Boophilus*) *microplus* (Canestrini, 1888) also called the Southern cattle tick is a serious pest of cattle in various tropical and subtropical countries of Africa and Latin America, as well as in northern and eastern Australia. This tick species represents one of the main constraints on the advancement of cattle production, as it has a direct parasitic action and is the vector of important pathogens. Parasitism by *R.* (*Boophilus*) *microplus* results in poor condition, weight loss, reduced meat and milk production, and potential transmission of *B. bovis*, *B. bigemina* and *Anaplasma marginale* (Estrada-Peña and Venzal, 2006).

Rhipicephalus (*Boophilus*) *microplus* is thought to have originated in south East Asia and has a pantropical distribution. In Africa, *R.* (*Boophilus*) *microplus* was restricted to the Malagasy region, south and east Africa. Except for extremely cold and dry areas, *R.* (*Boophilus*) *microplus* has extended its distribution range and is now present in all northern regions of Tanzania (Lynen et al., 2008). It is also known that large areas of neighbouring Kenya and Central Africa are climatically suitable for this tick (Sutherst and Maywald , 1985). In West Africa, *R.* (*Boophilus*) *microplus* was found for the first time in 2007 on dairy cattle in the Ivory Coast (Madder et al., 2007).

Rhipicephalus (*Boophilus*) *microplus* is a one-host tick that uses cattle as its common host and is usually only found on other animals if infested cattle are present in the same locality. For example, parasitism by *R.* (*Boophilus*) *microplus* on marsh deer (*Blastocerus dichotomus*) was associated with the presence of cattle in neighbouring farms. Other domestic and wild animals can be parasitized if crossing or sharing highly infested pastures (Szabo et al., 2007). This tick species has been found on humans, and even though their vectorial capacity deserves further investigation (Guglielmone et al., 2006), human cases of *B. bovis* has been reported in Africa (Rodriguez et al., 1984) and South America (Rios et al., 2003). Occurrence in a particular area is contingent upon the presence of cattle, medium to high humidity and a mean temperature higher than 14.5°C during most months of the year (Guglielmone, 1992).

Environmental factors have a remarkable influence upon the free-living cycle of *R.* (*Boophilus*) *microplus*. In suitable conditions, heavy rains, high grass stalks and high temperatures, the whole non-parasitic phase may be less than 3 weeks. Cold weather and scanty rainfalls are adverse factors for survival of *R.* (*Boophilus*) *microplus*. Some variation in duration of the parasitic life cycle are possible; often two generations per year are seen but in some tropical areas more than four generations per year can occur (Williams et al., 1985). Once the larvae are on the host, they wander freely in search of appropriate sites to attach, generally areas that are rich in vascularization, such as the inner side of the thighs, perineal region, dewlap, neck and anterior border of the ears (Nunez et al., 1985). When they have completed feeding, they remain attached to the host and moulting occurs in situ. The nymphs then feed on the same host and also remain attached. The final moult generates adults that feed on the same host and then change position for mating. Thus all three stages of any individual tick can occur on the same individual host, with a total feeding time of approximately 18 days (Walker et al., 2003). Studies on mating and gene transmission have however shown that, contrary to expectation, *R.* (*Boophilus*) *microplus* frequently settles on distinct individual cows, either as larvae seeking a new host, or as juvenile transferred during contact between cows. These findings are pivotal for understanding the epidemiology of disease caused by tick-borne micropathogens (Chevillon et al., 2007).

Rhipicephalus (*Boophilus*) *decoloratus* or the "blue tick" is the most widespread and frequent one-host cattle tick species in Africa. Cattle are the main host of *R.* (*Boophilus*) *decoloratus* but it also feeds on horses, donkeys, sheep, goats and wild ungulates. Cattle are probably the only maintenance host for this tick, and infestations of other hosts will only occur when a population of ticks is maintained by the presence of cattle. This tick species occurs in regions with savanna typically in grasslands and wooded areas used as cattle pasture. It is very widely distributed in suitable habitats throughout Africa, south of the Sahara. It tends to be absent from the drier areas of countries such as Namibia, South Africa and Botswana. In West Africa it occurs together with *R.* (*Boophilus*) *annulatus, R.* (*Boophilus*) *geigyi* and recently with *R.* (*Boophilus*) *microplus*; in East Africa and southern Africa it occurs together with *R.* (*Boophilus*) *microplus*. In southern Africa, larval hatching from eggs that have over-wintered is synchronized as the temperature rises in spring, and large numbers of larvae are present on the vegetation at this time. Because of the tick's short life cycle, several generations of larvae occur throughout the summer and into the cooler months of May and June. After the spring rise, the largest numbers of ticks are present on cattle in southern Africa during the summer

and autumn to early winter months. On wild herbivores in this region the highest burdens are usually recorded only in spring and, unless these animals are stressed, their late summer and autumn burdens remain low. North of the Equator, *R.* (*Boophilus*) *decoloratus* is most abundant on cattle during the rainy season or in autumn. *Rhipicephalus* (*Boophilus*) *decoloratus* transmits the protozoan *B. bigemina*, the bacteria *Anaplasma marginale* and *Borrelia theileri*, the cause of spirochaetosis in cattle, sheep, goats and horses. Heavy infestations of *R.* (*Boophilus*) *decoloratus* are likely to cause damage to hides and to reduce the rate of cattle growth (Walker et al., 2003).

Rhipicephalus (*Boophilus*) *annulatus* is a typical one-host tick of the *Boophilus* sub-genus. The discovery in 1893 in the USA that *Babesia* protozoa were transmitted from cow to cow by this tick species was the first time that such a route of transmission of a pathogen was described, and this was one of the factors that started the science of vector biology. Cattle are the main hosts of *R.* (*Boophilus*) *annulatus* but occasionally sheep, goats and wild ungulates can also support successful completion of the life cycle. The period of infestation of cattle is approximately three weeks. The life cycle can be completed in two months, and six generations per year are possible under conditions of continuous high temperature and humidity. In North Africa, where there are distinct summer and winter seasons, the tick's activity begins in late summer and extends from September to January with a peak in autumn (October). *Rhipicephalus* (*Boophilus*) *annulatus* survives mainly in areas with Mediterranean climate and savanna but in Sudan occurs in humid localities within steppe areas that have very hot dry seasons. This is mainly a tick of West and North Africa but it is found elsewhere such as south-east Sudan, Central African Republic and Democratic Republic of Congo. In the Americas this tick occurs in Mexico but has been eradicated from the USA. *Rhipicephalus* (*Boophilus*) *annulatus* is often found together with *R.* (*Boophilus*) *decoloratus*. This species of tick transmits the protozoans *B. bigemina* and *B. bovis*, it also transmits the bacterium *Anaplasma marginale* to cattle (Walker et al., 2003).

Rhipicephalus (*Boophilus*) *geigyi* has a much more limited distribution, and is restricted to the warmer and more humid portions of West Africa (Estrada-Peña et al., 2006) with scattered populations eastwards to Uganda (Walker et al., 2003). Cattle are the maintenance host of this tick but this tick species is also found on sheep, wild ungulates and dogs considerably less often. The life cycle can be completed in approximately two months and repeated generations can occur each year. This tick species occurs in savanna climatic regions with warm humid wooded grasslands and is probably most common when such areas are used as cattle pasture.

The disease relationships of this tick are poorly known, however *B. bovis* was found in eggs and larvae (Akinboade and Dipeolu, 1981).

Rhipicephalus (*Boophilus*) *kohlsi* is mainly found in the Middle East (Syria, Iraq, Israel, Jordan, western Saudi Arabia, and the Yemen). It is the only boophilid restricted to sheep and goats (and occasionally horses) (Estrada-Peña et al., 2006). The disease relationships of this tick are poorly known.

2. Dermacentor reticulatus

Dermacentor reticulatus (Fabricius, 1794) commonly called the "ornate cow tick" or the "marsh tick" is a large ornate tick. Adults feed on domestic and wild mammals such as dogs, cattle, deer, horses and occasionally humans (Dautel et al., 2006). The nymphs and larvae feed mainly on small mammals. It is a three-host tick (each stage feeding once on a separate host) that seems to prefer heavily wooded areas, grassland and pasture. One or two life cycles can be completed per year, with the main activity period for the adult in spring and a second peak in autumn (Hillyard, 1996). The distribution of *D. reticulatus* in North-Western Europe is sparse and localised (Hillyard, 1996). The geographic range of this species extends from France and the southwest United Kingdom through to central Asia. Its southern limit is the Mediterranean Sea, but here it is restricted to humid areas of medium altitude. Until recently, the French-Belgian border was considered to be the northern boundary of *D. reticulatus* in Western Europe (Heile et al., 2006) and within its entire endemic area, distribution of this tick species is considered to be highly focal (Gray et al., 2009). Recently, several reports have indicated that the geographical distribution of *D. reticulatus* is expanding. Over the last decade, *D. reticulatus* ticks have been collected from the environment in The Netherlands (Nijhof et al., 2007) and in different German states (Länder) (Dautel et al., 2006). Supportive evidence for a change in the distribution of *D. reticulatus* has been provided by identification of canine babesiosis in regions of Germany (Heile et al., 2006; Barutzki et al., 2007), Hungary (Sreter et al., 2005), Switzerland (Porchet et al., 2007) and the Netherlands (Nijhof et al., 2007) not previously associated with this disease. Indeed, *D. reticulatus* is the most important vector of canine babesiosis (*Babesia canis canis*) (Martinod and Gilot, 1991), it can also transmit protozoan parasites of horses, *Theileria* (*B.*) *equi* and *Babesia caballi* (Perez-Eid, 2007), and is also a vector of tick-borne encephalitis virus (TBE), *Rickettsia conorii*

(boutonneuse fever) (Hillyard, 1996), *Rickettsia slovaca* (Tibola) (Raoult et al., 2002), *Francisella tularensis* (tularemia) and *Coxiella burnetii* (Q-fever) (Estrada-Peña and Jongejan, 1999). A *Borrelia* sp. (probably *B. burgdorferi*) was identified in *D. reticulatus* in Saxony in Eastern Germany (Kahl et al., 1992). More recently the presence of *Anaplasma phagocytophilum* DNA was detected in *D. reticulatus* ticks isolated from a red deer in Southern Belgium (Wirtgen et al., 2011).

3. Ixodes ricinus

Ixodes ricinus (Linnaeus, 1758), the "castor bean tick" or "sheep tick" is the most frequent tick species in Western Europe. The geographic range of this tick extends from Iceland to central Asia and in the south to North Africa (Bouattour et al., 1999). It is a three-host tick that feeds on wild and domestic animals, and humans can also act as hosts. All three stages can parasite medium to large sized hosts such as sheep, cattle, horse, dog and deer. The larvae and nymphs, however, usually feed on small mammals such as rodents or birds (Hillyard, 1996). *Ixodes ricinus* needs a humid environment and adequate temperature, commonly deciduous woodland, but can also be found on heathland or grassland with a high vegetative mat. Activity period is influenced by temperature and host availability. A primary peak occurs between April and June, and a second peak may occur in autumn. These peaks are usually separated by a period of low level of activity in summer. In winter, with temperatures commonly found in Europe, activity drops to nil (Hillyard, 1996). The development of *I. ricinus* takes typically two or three years, so is relatively long compared to most other tick species (Fain, 1990). *Ixodes ricinus* is a major vector of important diseases; it can transmit *Babesia divergens*, *Babesia* sp. EU1 and *B.* (*T.*) *microti* in Europe. It is also the vector of *Borrelia burgdorferi* (Lyme disease), *Rickettsia conorii* (boutonneuse fever), *Coxiella burnetii* (Q-fever), *Francisella tularensis* (tularemia), tick-borne encephalitis (TBE) and *Anaplasma phagocytophilum*: a potentially zoonotic bacterium which can cause Human Granulocytic Anaplasmosis (HGA) (Hillyard, 1996).

4. Ixodes hexagonus

Ixodes hexagonus is a nest-dwelling species known to be a parasite of the European hedgehog (*Erinaceus europaeus*) but which can occasionally be found on carnivores of the family Mustelidae or red foxes (*Vulpes vulpes*) as well as on companion animals such as dogs and cats (Arthur, 1963; Pfaffle et al., 2011). This tick is well distributed around Europe and is widely distributed in Belgium. *Ixodes hexagonus* can harbour various pathogens such as *Borrelia* spp., *A. phagocytophilum* (Skuballa et al., 2007; Skuballa et al., 2010; Silaghi et al., 2012), TBE (Krivanec et al., 1988) and probably *Rickettsia helvetica* (Nijhof et al., 2007), *Theileria annae* and *B.* (*T.*) *microti* (Walter, 1982; Camacho et al., 2003).

5. Ixodes scapularis

Ixodes scapularis (Say, 1821) is also called the "blacklegged tick" or the "deer tick" and is mainly found in the United States of America (USA) but is also present in Canada and Mexico. Distribution of this tick species seems to be expanding, the major limiting factors for population establishment being temperature and ambient humidity. The adult stage feeds on white-tailed deer (*Odocoileus virginianus*), cattle, dogs, and certain other medium-to-large sized mammals. White-tailed deer are an important host of adult *I. scapularis* and deer eradication generally results in significant reduction in immature *I. scapularis* populations in a given area (Duffy et al., 1994; Elias et al., 2011). However, the fact that deer eradication did not eliminate *I. scapularis* points to a role for other mammals as potential hosts of adult *I. scapularis.* Immature *I. scapularis* parasitize a wide variety of mammalian, avian, and reptilian hosts. Humans are occasionally parasitized by all stages of *I. scapularis* (Keirans et al., 1996). *Ixodes scapularis* has a life cycle that spans *2* years in northern populations, nymphs appearing early in spring and summer (May and June) (Spielman et al., 1984). Southern populations may have a generation times as short as 1 year, and nymphs and larvae may be found questing from January through September (Durden et al., 1996). These differences between populations reflect host availability rather than preference, transient availability of *I. scapularis* hosts serves to compress nymphal populations in space and time in northern populations, increasing the likelihood of human/tick interactions. *Ixodes scapularis* is the main vector of Lyme disease (*Borrelia burgdorferi*) in the USA and Canada.

It is also the major vector of *B.* (*T.*) *microti*, the agent of rodent and human babesiosis in the USA. Under laboratory conditions *I. scapularis* can transmit the agent of deer babesiosis, *B. odocoilei* (Waldrup et al., 1990). The etiologic agents of tularemia, *Francisella tularensis* and anaplasmosis, *Anaplasma marginale*, have been identified as naturally occurring in *I. scapularis* (Keirans et al., 1996). Recent analysis suggests that *I. scapularis* is a vector of the agent of human granulocytic anaplasmosis in the upper midwestern United States (Bakken et al., 1994). In addition to transmitting pathogenic agents to animals and humans, the bite of female *I. scapularis* can cause tick paralysis (Keirans et al., 1996).

J. Domestic and wild mammalian hosts of *Babesia* spp.

Host specificity

Host specificity concerns, primarily, specificity displayed by a *Babesia* species for a particular vertebrate host species under natural conditions. To date, about 100 species of *Babesia* have been described based on the morphology of intraerytrocytic stages detected in mammals. However, the pleomorphism observed in cells of different mammalian species and individuals, together with the results of some molecular studies, make some of these descriptions questionable. Some species of *Babesia* are considered to be less host specific than previously believed. It is likely that the number of valid species will decrease as more information becomes available (Hunfeld and Brade, 2004). Despite this trend towards rationalisation of classification, *Babesia* spp. with novel characteristics and phylogeny have recently been identified as pathogens for animals or humans around the world (e.g. WA1, MO1, CA1 to CA4, EU1) (Herwaldt et al., 1996; Herwaldt et al., 1997; Herwaldt et al., 2003).

1. Bovine *Babesia* species

a) In temperate climate regions

Babesia divergens and *Babesia major* are the two species confirmed to infect cattle in Europe and also in North Africa (Bouattour et al., 1999), with *B. divergens* being by far the most common. They are transmitted to the vertebrate host by *Ixodes ricinus* and *Haemaphysalis punctata*, respectively.

Babesia divergens is the causal agent of a widespread cattle disease known as "red water fever" and can also cause a dangerous zoonosis. It is a small pearshaped *Babesia* with an obtuse angle (divergent), generally located at the periphery of the erythrocyte. Adult female *Ixodes ricinus* acquire the infection when feeding and transmit transovarially to the larvae for two generations at least. This means that infection in ticks can persist for at least 4 years in the absence of cattle.

Babesia divergens exhibits low host specificity *in vitro*. Experimentally, *B. divergens* can infect several species: monkeys, mouflon, deer…but the only laboratory animal that has been found to be fully susceptible is the Mongolian gerbil, *Meriones unguiculatus*.

The significance of *B. divergens* for livestock industry in Europe is almost certainly underestimated, as is the possibility of human infection. A decrease in disease outbreaks, probably due in part to the introduction of effective prophylactic treatment and live vaccination in some countries (Gray et al., 1989), has been reported. However, many of the most effective drugs against *B. divergens* have since been withdrawn because of safety or residue problems (Zintl et al., 2003) and only one drug is licensed for use in Europe (see above).

In size *Babesia major* is between the large and the small *Babesia* spp. with an acute angle and central position in the erythrocyte. Adult females of *Haemaphysalis punctata* (*Boophilus calcaratus* in Russia) acquire infection and transovarial transmission occurs over three generations. *Babesia major* is considered less pathogenic than *B. divergens*, although if disease due to *B. major* occurs, clinical signs, diagnostic tests and treatment are similar.

b) In sub tropical and tropical regions

Babesia bovis and *B. bigemina* are responsible for a huge number of tropical babesiosis cases, globally. These parasites are endemic in Southern Europe, Africa, South America, Asia and Australia. Generally, both parasites have the same distribution but in Africa *B. bigemina* is more widespread than *B. bovis* (Bock et al., 2004).

Babesia bovis is a small species, frequently signet ring shaped or in pairs with an obtuse angle and a central peripheral position in the erythrocyte. *Babesia bovis* is acquired by adult female ticks of mainly *R.* (*Boophilus*) *microplus* and is transovarially transmitted to larval offspring. *Babesia bovis* causes one of the most important tick-borne diseases in tropical regions with enormous economical repercussions, not only from mortality or production losses but also throught its impact on the international cattle market (Bock et al., 2004). In addition specific nervous symptoms can be found in case of *B. bovis* infection.

Babesia bigemina is a large *Babesia*, pairs of pearshaped organisms within the red blood cells forming a sharp, acute angle. In Africa, *B. bigemina* is transmitted by *R.* (*Boophilus*) spp. and other *Rhipicephalus* spp. while in Australia and South America it is *R.* (*Boophilus*) *microplus*. In Europe, *B. bigemina* was mainly reported in the Southern part whereas cases were also reported in Switzerland (Hilpertshauser et al., 2007) and Austria (Edelhofer et al., 2004), and were transmitted by *Haemaphysalis punctata*. Acute cases are usually not as severely affected as those with *B. bovis* infections. No cerebral involvements are present and recovery is usually rapid and complete. However, sometimes the disease can develop very rapidly with severe anaemia (Bock et al., 2004).

2. Small ruminants *Babesia* species

Babesia ovis, *Babesia motasi*, *Babesia crassa* and several unrecognized *Babesia* species have been described as being infective for small ruminants. *Babesia motasi* is a large *Babesia* species found in sheep and goat in Europe, the Middle East, Russia, Vietnam, North Africa and probably in Central Africa (Rwanda). This species is transmitted by *H. punctata*, *H.*

otophila, *Rhipicephalus bursa* and *Dermacentor silvarum*. The pathogenicity is variable and infections range from acute to chronic. Sheep seems more sensitive than goat and with variation between breed. This parasite appears to be of moderate virulence and the acute form of the disease is characterized by anorexia, fever, anaemia, icterus and haemoglobinuria. Other features include abdominal pains, diarrhoea, prostration and death can occur. The chronic form of the disease is characterized mainly by emaciation but coughing and oedema are also characteristic.

Babesia ovis is a small *Babesia* species which infect sheep, goat and wild sheep in Europe, the Middle East and in most of the tropical and subtropical regions. This parasite is transmitted by *Rhipicephalus* spp. and *I. persulcatus* and is highly pathogenic especially in sheep with frequent mortality.

A number of unclassified *Babesia* parasites infecting small ruminants have recently been reported in China. One species is closely related to *B. motasi* (including different geographic isolates such as *Babesia* sp. BQ1 (Lintan), *Babesia* sp. BQ1 (Ningxian), *Babesia* sp. Liaoning, *Babesia* sp. Tianzhu, and *Babesia* sp. Hebei), and another, based on phylogenetic analysis, is *Babesia* sp. Xinjiang (Guan et al., 2010).

A species involved in the first human case in Korea, namely *Babesia* sp. KO1, showed a sequence of the 18S rRNA gene closely related to a *Babesia* spp. isolated from sheep in China (*Babesia* sp. Hebei) (Kim et al., 2007).

3. Cervine *Babesia* species

Interest in “deer babesiosis” emerged recently with the identification of potentially zoonotic species in wild animals. Three species of *Babesia* have been described in European wild cervids: *Babesia* sp. EU1 (proposed nomenclature, *Babesia venatorum), B. divergens* and *Babesia capreoli*. These three species share the same tick vector namely *Ixodes ricinus*. The role of wild fauna as potential reservoir hosts for zoonotic *Babesia* species is of great importance.

Babesia sp. EU1 was first discovered in human and has also been identified in roe deer (*Capreolus capreolus*), but not yet in red deer (*Cervus elaphus*) (Duh et al., 2005). Discrimination of this species was mainly based on molecular analysis of the 18S rRNA gene. The sequence derived from EU1 showing a 31 base pair (bp) difference compared to the *B. divergens* sequence and a 29 bp difference with the *B. odocoilei* sequence (Herwaldt et al., 2003). Phylogenetic analyses indicated that *Babesia* sp. EU1 is most closely related to *B. odocoilei*, a white-tailed deer parasite found in the USA, and was placed in a sister group relative to *B. divergens*. However, analysis by morphology and serology failed to distinguish these two species, even though multiplication differed in gerbils, EU1 being unable to grow in this host (Herwaldt et al., 2003).

Babesia divergens experimentally has a wider host range than attributed to natural infection. Roe, fallow (*Dama dama*) and red deer can be experimentally infected with *B. divergens* and show milder clinical signs and a low parasitaemia (Duh et al., 2005). Natural infection of roe, red deer (Duh et al., 2005) and reindeer (*Rangifer tarandus*) (Langton et al., 2003) has been proposed, even though identification based on a fragment of the 18S rRNA gene remains questionable.

Babesia capreoli infection in roe deer (Enigk and Friedhoff, 1962), red deer (Adam et al., 1976) and sika deer (*Cervus nippon*) (Gray et al., 1990) has been reported. Morphology and serology could not differentiate *B. divergens* and *B. capreoli* and discrimination of *B. capreoli* was based on the fact that it could not infect splenectomized calves or gerbils. Recently, *in vitro* culture demonstrated that specimens derived from roe deer were unable to grow in cattle, human or sheep erythrocytes. Moreover, molecular analyses identified systematically 3 bp differences in the 18S rRNA gene and it was concluded that *B. capreoli* and *B. divergens* were separate species (Malandrin et al., 2010).

4. Canine *Babesia* species

The first case of canine babesiosis was observed in the Cape Colony in 1893 (Penzhorn, 2011). Since then, different species and subspecies of canine *Babesia* have been identified, based on differences in the tick vectors, morphology, gene sequences and clinical signs.

Morphologically, they are classified in two groups namely, large and small Babesias, both with a fairly large distribution (Schoeman, 2009). The severity of the clinical symptoms varies from peracute to subclinical, depending on *Babesia* species, age and immunological status of the host.

Babesia canis, a large *Babesia* species, is comprised of three subspecies: *B. canis canis*, *B. canis rossi* and *B. canis vogeli* (Uilenberg et al., 1989). *Babesia canis canis* is the main species found in Europe and is transmitted by *Dermacentor reticulatus*. This parasite causes a variety of symptoms such as lethargy, anorexia, fever, jaundice, anaemia and thrombocytopenia.

Babesia canis rossi transmitted by *Haemaphysalis elliptica* in sub-Saharan Africa, predominantly in South Africa, is the most virulent subspecies causing acute disease with fever, depression, anaemia, and splenomegaly. Puppies and immature dogs are also seriously affected (Solano-Gallego et al., 2008; Penzhorn, 2011).

Babesia canis vogeli infections transmitted by *Rhipicephalus sanguineus* in North Africa, the Middle East and India are mainly unapparent or present moderate symptoms in adult dogs; however, in puppies severe disease can occur (Schoeman, 2009). An unnamed large *Babesia* sp. was also isolated from dogs in the USA (Birkenheuer et al., 2004).

Small *Babesia*-like spp. of dogs are represented by at least 5 different taxa: *B. gibsoni sensu stricto*, *B. conradae* isolated in southern California (Kjemtrup et al., 2006), *B.* (*T.*) *microti*-like (Zahler et al., 2000), *Theileria annae* (Camacho et al., 2001) and an unnamed *Theileria* sp. (Matjila et al., 2008). *Babesia gibsoni* occurs principally in the Middle East, southern Asia, Japan, North Africa, and South America and is considered as emerging in the USA. This small *Babesia* sp. is found as single or annular bodies and is transmitted by *Haemaphysalis bispinosa* and *Haemaphysalis longicornis*. The recognition of these small parasites requires good staining technique and considerable practice. *Babesia gibsoni* infection follows most commonly an acute course characterized by fever, lethargy, thrombocytopenia, haemolytic anaemia, splenomegaly and lymphadenopathy. These parasites are very difficult to clear with conventional therapy and dogs usually become chronic carriers or present recurrent episodes of acute babesiosis. The first treatment that has been shown to be effective against *B. gibsoni* is a combination of atovaquone and azithromycin (Schoeman, 2009).

5. Equine *Babesia* species

Equine babesiosis occurs in temperate and tropical regions and is caused by *Babesia caballi* and *Theileria* (*Babesia*) *equi* transmitted by ticks within the genera *Dermacentor, Hyalomma* and *Rhipicephalus*. The clinical signs of equine piroplasmosis are often nonspecific, and the disease can easily be confused with other conditions. Piroplasmosis can occur in peracute, acute and chronic forms. The acute cases are more common, and both species can cause characteristic babesiosis symptoms such as fever, anaemia, icterus and haemoglobinuria. In subacute cases, affected animals show loss of weight, and fever is sometimes intermittent. Additional features can be seen include sweating, mild colic, lacrimation, incoordination, cardiac murmurs, and subcutaneous oedema around the head and eyelids. Mild oedematous swelling of the distal part of the limbs sometimes occurs. Chronic cases usually present nonspecific clinical signs such as mild inappetence, poor performance and a drop in body mass (OIE, 2008). As in cattle, foals are more resistant to infection and less likely to develop clinical disease.

Babesia caballi is a large species with infected cells more abundant in the capillary blood vessels and this can create obstruction of these vessels.

Theileria equi, formerly named *B. equi*, is a small piroplasm. It was reclassified within the *Theileria* family following description of schizogony in lymphocytes (Schein et al., 1981) and the absence of transovarial transmission in the tick vector. Infections by *T. (B.) equi* are usually clinically more severe than those of *B. caballi,* and present an elevated parasitaemia and require higher dosages of specific drugs as imidocarb.

6. Zoonotic aspects of babesiosis

Babesiosis is recognized as a potentially emerging disease in humans but is probably better described as under-diagnosed (Hunfeld and Brade, 2004). Human infections have been reported in Europe, the USA, Japan, Taiwan, Korea, China, Egypt, Brazil, India and South Africa (Bush et al., 1990; Humiczewska and Kuzna-Grygiel, 1997; Shih et al., 1997; Saito-Ito et al., 2000; Marathe et al., 2005; Kim et al., 2007; El-Bahnasawy and Morsy, 2008; Qi et al., 2011). Babesiosis is increasingly gaining attention and is a growing public health concern, mainly in the USA (Hunfeld et al., 2002; Gray et al., 2010). Different species have been identified as potentially zoonotic (Table 3), and at least four different groups of *Babesia* spp. have been recognized as a cause of human babesiosis. These are 1) *B. divergens*, *B. divergens*-like parasites and *Babesia* sp. EU1; 2) *B.* (*T.*) *microti*, a natural parasite of microtine rodents such as white-footed mouse (*Peromyscus leucopus*) in USA or field voles (*Microtus agrestis*) and shrew (*Sorex araneus*) in Europe (Bown et al., 2011); 3) *Babesia* sp. WA1, recently named *Babesia duncani* and morphologically similar to *B.* (*T.*) *microti* but phylogenetically distinct and 4) a single species, *Babesia* sp. KO1 from Korea that seems to be related to a *Babesia* of sheep (Gray et al., 2010). Humans acquire disease mostly through tick bites or via blood transfusion or, exceptionally, by transplacental transmission (Esernio-Jenssen et al., 1987). Elderly people, splenectomized people or people with depressed cell mediated immunity are associated with a higher risk of contracting symptomatic babesiosis. An increasing number of HIV and immuno-compromised patients may provide a predicted boost in the number of human cases (Hunfeld et al., 2008).

***Babesia* species**	**Distribution of clinical cases**	**Reservoir host**
B. divergens	Europe	Cattle
B. divergens-like	USA, Portugal	Unknown
Babesia sp. EU1 (*B. venatorum*)	Austria, Italy, Germany	Deer
B. (*T.*) *microti*	USA, Japan, Taiwan, Germany	Rodents
B. duncani (*B.* sp. WA1), *Babesia* sp. CA1-CA4	USA	Unknown
Babesia sp. KO1 (ovine *Babesia*-like)	Korea	Sheep (?)

Table 3: Potentially zoonotic *Babesia* spp., their reservoir hosts and clinical cases distribution.

In humans, clinical features vary substantially from asymptomatic to life threatening, depending on the immune status of the patient and the parasite species involved (Hunfeld et al., 2008). In general, patients of all ages including children could be affected, but elderly individuals are most at risk. Nonspecific clinical presentation can be easily confused with malaria; high fever, intense sweats, headaches, shaking chills, myalgia, lumbar and abdominal pain are included in the list of symptoms (Gray et al., 2010). Jaundice can develop due to severe haemolysis; vomiting and diarrhoea may be present (Zintl et al., 2003).

Heavy infections, often due to *B. divergens*, induce an acute illness with haemoglobinuria. These symptoms mainly occur in immuno-compromised or elderly patients, although cases have been reported recently in immuno-competent patients (Martinot et al., 2011). In the most severe cases, patients develop shock-like symptoms with respiratory, cardiac, renal or hepatic failure due to haemolysis and the host immunological response (Figure 20). Pulmonary complications are most common in people experiencing severe babesial infection (Krause et al., 2007). The few reported cases attributed to the newly described species, *Babesia* sp. EU1 have shown similar but milder symptoms (Hunfeld et al., 2008). Infections due to *B.* (*T.*) *microti* show a wider range of symptoms; the disease seems to be milder, with asymptomatic patients or a mild flu-like illness that is rarely diagnosed. Severe cases occur preferentially in splenectomised and elderly patients or individuals with immunosuppressive treatment (Gray et al., 2010). Immuno-compromised patients are also likely to develop persistent relapsing disease, despite treatment (Krause et al., 2008). In HIV patients, fever may occur for more than 4 weeks and show high parasitaemias (>30%) with possible relapse after treatment.

Infections with the newly recognized *B. divergens*-like parasite that occurred in Washington State, and a case of babesiosis in Korea due to a *Babesia* parasite closely related to ovine *Babesia* spp., provisionally named KO1, presented a more benign clinical course and the patients recovered after treatment (Hunfeld et al., 2008).

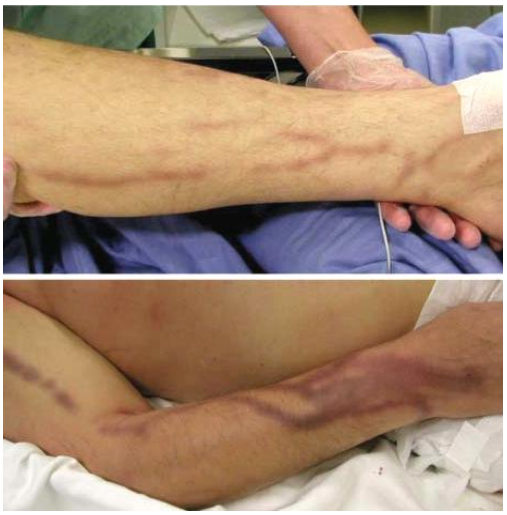

Figure 20: Lesions of a patient infected with *B. divergens* in Finland. Dark streaks on the legs and arms, probably caused by a massive intravascular haemolysis (Haapasalo et al., 2010).

a) In Europe

The first case of human babesiosis was described in 1956 (Skrabalo and Deanovic, 1957) in a splenectomised Yugoslavian farmer and was probably due to the cattle parasite, *B. divergens*. Since then, approximately 40 cases have been reported in Europe (France, Great Britain, Ireland, Spain, Portugal, Switzerland, Sweden), mainly due to *B. divergens*.

The first cases associated with *Babesia* sp. EU1 (*Babesia venatorum*) were discovered in Austria and Italy in two patients respectively afflicted with Hodgkin's disease or undergoing chemotherapy (Herwaldt et al., 2003). A subsequent case described in an individual in Germany was reported in 2007 (Häselbarth et al., 2007). The Latin name "venatorum" is translated as "of the hunters" and was chosen because of the hunting activities of the first two patients. The parasite was defined as a new species based on a 31 bp difference in the 18S rRNA gene sequence compared to *B. divergens* and the inability to grow in gerbils. However, degree of sequence diversity in a particular gene that is required to identify and discriminate a new or different species is controversial, and it is unknown if *Babesia* sp. EU1 and *B. divergens* could undergo sexual recombination if present in the same tick blood meal. *Babesia* sp. EU1 has been identified in roe deer (Duh et al., 2005; Bonnet et al., 2007) and in *Ixodes ricinus* (Duh et al., 2005; Casati et al., 2006) with evidence of transstadial and transovarial transmission of the parasite within the tick (Bonnet et al., 2007).

Although human infections with *B.* (*T.*) *microti* are more common in the USA, this species is known to be present in *Ixodes ricinus* and serological evidence of human *B.* (*T.*) *microti* infection has been recorded in different European countries (Hunfeld et al., 2002; Pancewicz et al., 2011). A case of autochthonous *B.* (*T.*) *microti* infection has been reported in a patient afflicted by an acute myeloid leukaemia in Germany (Hildebrandt et al., 2007). In Belgium, a study has been conducted in 1981 (Jadin and Giroud) from patients suspected of rickettsiosis and a seroprevalence of 7.8% has been shown for *B.* (*T.*) *microti* and 8.08% for another rodent species, *Babesia rodhaini*, which cause an acute fulminating lethal disease in mice. Moreover, a clinical case was suspected in a non-splenectomised 40 year-old patient, with recurrent hyperthermia, asthenia and weight losses. Serology of the patient gave positive results for *Rickettsia conorii* (the agent of boutonneuse fever), *B.* (*T.*) *microti* and *B. rodhaini*.

b) In the USA

In the USA, the first case of human babesiosis was diagnosed in California in 1966 (Scholtens et al., 1968). Human cases in the USA are mainly attributed to the natural parasite of microtine rodents, *B.* (*T.*) *microti*, and the first documented human case was reported on Nantucket Island (Western et al., 1970). Since then, hundreds of human babesiosis cases have been reported with an increasing occurrence each year. This high frequency is in contrast to that of all other regions in the world. This may be due to a higher awareness of the clinical manifestations of the disease, a better availability of diagnostic tools (Hunfeld et al., 2002) or an increased virulence of *B.* (*T.*) *microti* strains found in the USA.

Babesia (*Theileria*) *microti* cases occur in both intact and asplenic patients. Transmission to the human host is mostly attributed to tick bites of the most common vector, *Ixodes scapularis*. Unlike other classical *Babesia* species, transovarial transmission is not present and this together with the occurrence of schizogony in lymphocytes have led some taxonomists to recommend classification of this parasite in the genus *Theileria* (*Theileria microti*) (Uilenberg, 2006).

Blood transfusion seems to be a common route for transmission of *B.* (*T.*) *microti* to humans and increased attention of babesiosis as a public health concern occurred with the diagnosis of the organism as a cause of death in transfusion recipients. Strategies to minimize the risk of transmission via blood should be implemented but management to ensure a *Babesia* free

source is not particularly easy. The cost-benefit of different approaches has to be taken into account and the geographical limitation of the disease to the Northeast and Midwest regions of the USA has hindered the implementation of a universal screening program for donated blood. A lack of sensitivity and specificity of tests and surveys designed to assess the risk of contamination is also a major restraint (Leiby, 2011).

A novel species of *Babesia* associated with human infection has been identified in Washington State and California, as *Babesia* sp. WA1-WA3 (Quick et al., 1993; Herwaldt et al., 1997) and CA1-CA6 (Persing et al., 1995; Kjemtrup et al., 2002). WA1 and CA5 parasites were used as a reference to describe this new species subsequently called *Babesia duncani* (Conrad et al., 2006). The identity of the tick vector(s) and reservoir host(s) of *B. duncani* are unknown but its taxonomy incriminates wild ruminants as natural reservoirs of infection (Gray et al., 2010). Clinical cases due to *B. duncani* are rare but identification of infection in spleen intact patients and the possibly of transmission by blood transfusion, suggest that there are many asymptomatic infections (Conrad et al., 2006; Gray et al., 2010).

Three cases of human babesiosis were identified in Missouri (Herwaldt et al., 1996), Kentucky (Beattie et al., 2002) and Washington States (Herwaldt et al., 1997) and attributed to *B. divergens*-like parasites. These reports suggest the presence of *B. divergens* in the USA but as for *B.* (*T.*) *microti* in Europe, *B. divergens* is not recognized as a common source of clinical cases in the USA.

c) Associated tick-borne pathogens

Ticks that act as vectors for *Babesia* spp. are known to be frequently infected by one or more additional potentially zoonotic pathogens, such as *Borrelia burgdorferi* sensu lato, *Anaplasma phagocytophilum, Coxiella burnetii* or *Rickettsia* spp. (Tokarz et al., 2010; Hildebrandt et al., 2011a). Multi-infections could potentially influence the transmission dynamics and epidemiology of tick-borne disease (TBD) by affecting tick behavior and survival (Ginsberg, 2008). Moreover, concurrent TBD infections can also increase the severity and duration of the sickness (Krause et al., 1996).

Co-morbidity with *Borrelia burgdorferi,* the etiologic agent of Lyme disease, and *Anaplasma phagocytophilum* causing Human Granulocytic Anaplasmosis (HGA) is well known (Krause et al., 2002). *Anaplasma phagocytophilum* have been recorded in cattle, sheep, horse, dog,

deer and humans and can induce clinical manifestations such as high fever, abortions and secondary infections (Stuen, 2007). Human symptomatic infection seems to be rare in Europe, despite a range of seroprevalence between 6.2 and 21% (Cochez et al., 2011a). This bacterium has been described in almost all Europe and recently in cattle and humans in Belgium (Guyot et al., 2011; Cochez et al., 2011a). Babesiosis and HGA could be difficult to differentially diagnose because both show non-specific flu-like symptoms during the early stages of disease. Fortunately, acute Lyme disease can be diagnosed via the classical erythema migrans (Figure 21) but this pathognomonic sign is sometimes absent and consequently such cases can be confused with babesiosis (Krause et al., 2002). Further laboratory diagnosis tests for tick-borne pathogens on patients with prolonged flu-like illness that fail to respond to anti-*Borrelia* treatment (Krause et al., 2002) are required. Therefore, in order to gain detailed insight into the epidemiology of tick-borne infection and disease, it is necessary to consider the total pathogen population profile that can be transmitted to particular hosts in an endemic area.

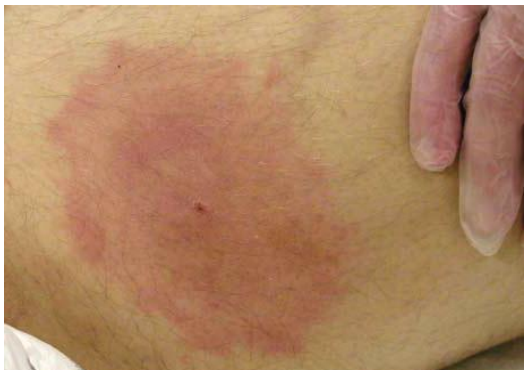

Figure 21: Erythema migrans in a patient co-infected with *B. divergens* and *Borrelia* sp. (Haapasalo et al., 2010).

II. Objectives

The research carried out for this PhD thesis focused on *Babesia* spp. found in diverse vertebrate hosts, environments and various tick species that act as primary vectors. The general theme of the study is comprised of several research areas and has been divided in three different sections, each with specific objectives.

In the first section, epidemiological, molecular and serological tools were used to investigate the complex relationship between *Ixodes ricinus* (the tick species most commonly found in Belgium), *Babesia* spp. pathogens and different vertebrate hosts such as dogs and cats (Study 1), cattle (Study 2) and wild cervids (Study 3). Additionally, the occurrence of co-infection of *I. ricinus* with *Babesia* spp. and *A. phagocytophilum* was investigated.

In the second section, molecular and epidemiological tools were used in two very different situations to study the presence and distribution of important tick species with respect to transmission of *Babesia* spp. and their potential emergence or spread. Geographical vector expansion was investigated for *D. reticulatus* in Belgium and *R.* (*Boophilus*) *microplus* in West Africa because for both situations the potential risk of tick-borne diseases emerging in a previous uninfected area was suspected. In the West African study (Study 4), a PCR-RFLP technique was used in order to differentiate tick species of the genus *Rhipicephalus* (*Boophilus*) namely *R.* (*B.*) *microplus*, *R.* (*B.*) *decoloratus*, *R.* (*B.*) *annulatus* and *R.* (*B.*) *geigyi* collected from cattle in Africa. The first of these species has been recently identified in West Africa and represents a major threat for cattle breeding in this part of the world and elsewhere. Differentiation from other species of this genus is very difficult and time consuming. In the Belgian study (Study 5), classical epidemiological tools (flagging and microscopy) were used in order to determine the presence and potential distribution of *Dermacentor reticulatus* in Belgium, an important tick species that feeds as adults on dogs, horses and ruminants. A preliminary study on the vectorial capacity of this tick for *Babesia* spp. was also carried out.

Finally, a retrospective serological survey was performed on a preselected bank of sera from human patients in Belgium with history of tick bite and a suspicion of tick-borne diseases. The results were used to evaluate the potential exposure to *Babesia* spp. in this at risk group of the human population (Study 6).

III. Part I

A. Article 1: First molecular evidence of potentially zoonotic *Babesia microti* and *Babesia* sp. EU1 in *Ixodes ricinus* ticks in Belgium

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Key Words: *Babesia microti*, *Babesia* sp. EU1, Belgium, Emerging zoonotic infection, *Ixodes ricinus*, PCR, Ticks.

1. Introduction

Babesiosis is a tick-borne disease caused by different species of intraerythrocytic protozoa of the genus *Babesia*. It is a common infection in animals but receiving increasing attention as an emerging tick-borne zoonosis in Europe (Hunfeld and Brade, 2004; Meliani et al., 2006; Vannier and Krause, 2009). Human babesiosis, a malaria-like disease, has broad spectrum of clinical presentations from a subclinical infection to death according to age, immunocompetence and co-morbidity. Asplenic or immunocompromised patients represent the most susceptible groups. In Europe, human infections acquired from ticks and also from blood transfusions (Krause, 2003) are rarely detected but potentially fatal (Homer et al., 2000; Hunfeld et al., 2008). Man is an accidental host, and before the molecular era, most European human infections were attributed to *Babesia divergens*, the causative agent of cattle babesiosis in temperate countries (Herwaldt et al., 2003). More recently, a new candidate species designated *Babesia* sp. EU1 (for Europe 1 and proposed as *B. venatorum*) was described for the first time in two asplenic patients (Herwaldt et al., 2003) and also in roe deer

in Europe (Duh et al., 2005; Bonnet et al., 2007). This species seems to be closely related to *B. odocoilei* (a white-tailed deer parasite) and phylogenetically in a sister group with *B. divergens* (Herwaldt et al., 2003).

Babesia (*Theileria*) *microti*, a parasite of small mammals, is the main agent of human babesiosis in North America. This parasite also occurs in ticks in Europe (Duh et al., 2003; Hartelt et al., 2004; Nijhof et al., 2007). However, only two cases of human babesiosis attributed to *B.* (*T.*) *microti* have been reported in Europe (Meer-Scherrer et al., 2004; Hildebrandt et al., 2007) and one of these cases remains controversial (Gray, 2006). In the United States, other zoonotic *Babesia* spp. and *Babesia*-like pathogens have been identified using molecular tools as WA1 (for Washington 1) (Quick et al., 1993) and MO1 (for Missouri 1) (Herwaldt et al., 1996). In Belgium, there is little information regarding the prevalence of the different species of *Babesia* and their potential zoonotic impact. *Babesia divergens* is endemic in the southern part of the country and was considered absent from other parts of Belgium (Saegerman et al., 2007), although, recently, an outbreak of cattle babesiosis was reported in Vlaams Brabant (Central Belgium). Introduction was probably through importation of infected cattle from the south of Belgium (Everaert et al., 2007). As far as we are aware, human babesiosis due to *B. divergens* has never been reported in Belgium.

In Belgium, *Ixodes ricinus* is the most prevalent tick species and an important vector of diseases such as babesiosis, borreliosis, and anaplasmosis. It is a competent vector of both *B.* (*T.*) *microti* and *Babesia* sp. EU1 (Foppa et al., 2002; Becker et al., 2009; Bonnet et al., 2009). Dogs and cats are close to humans and have often been proposed as effective sentinel animals to assess the risk of human tick-borne diseases (Olson et al., 2000; Duncan et al., 2005).

This study was designed to assess by means of molecular tools the presence and the infection rate of potentially zoonotic *Babesia* species in different species of ticks found on dogs and cats in Belgium.

2. Materials and Methods

From April 2008 to April 2009, a nationwide survey was performed in Belgium. In the 25 veterinary districts, three companion animal practices were selected by stratified randomization. Ticks from cats and dogs, randomly submitted to the 75 veterinary practices, were collected and preserved in 80% alcohol. The ticks were morphologically identified up to stage and species level using a standard key for morphological identification (Arthur, 1963).

The sex and repletion level were recorded for adults. For each animal enrolled in the study, information was available via a questionnaire completed by the veterinarian about location, date of collection, animal description (dog-cat, age, sex, and breed), and life habits. Ticks collected from dogs and cats with a recent travel history (3 weeks or less before submission) were excluded from the trial. One tick per animal was selected, but when several tick species were present on a given animal, one tick of each species was randomly selected for further analysis. Tick DNA extraction was performed using a protocol with proteinase K (20mg/mL) (Boom et al., 1990).

To detect false-negative results due to polymerase chain reaction (PCR) inhibition and to validate the efficiency of the DNA extraction, an additional PCR targeting a 325-bp DNA fragment corresponding to the tick 16S rRNA gene was included. This PCR was set up using 16S+1 and 16S-2 primers (Baumgarten et al., 1999). Only positive samples were further analyzed for the presence of *Babesia* spp. Repletion level was recorded and potential inhibition due to engorgement of the ticks and the effect of DNA dilution on results was assessed. To do this, 335 DNA samples were chosen randomly and diluted 1/10. Any negative sample was then tested either neat or after 1/100 dilution. Remaining negative samples were again diluted to 1/1000. DNA concentration was recorded. A *Babesia* spp. genus-specific PCR was developed according to Casati et al. (2006) using BJ1 and BN2 primers and based on the amplification of a 411–452-bp fragment of the multicopy 18S rRNA gene. To assess the specificity and to validate this PCR method, the following positive controls were used: *Babesia canis* extracted from canine blood (kindly supplied by ENV Lyon), *B. divergens* extracted from cattle blood and *Babesia* sp. EU1 from deer blood (both supplied by ENV Lyon), and *Babesia* sp. EU1 (supplied by Zurich University). Whenever a tick was found positive for *Babesia* spp., the other ticks found on the same animal were also examined.

To assess the sensitivity of the *Babesia* spp. PCR, the DNA concentration of an extract originating from a *B. canis* strain (ENV Lyon) was measured by spectrometry (Nanodrop; Thermo Scientific). The extract was then serially diluted 10-fold with sterile water. Tick DNA samples positive for *Babesia* spp. were sequenced from PCR products and after cloning. The PCR products were purified using MSB-Spin PCRapace kit (Invitek) and cloned with Topo TA Cloning Kit for sequencing (Invitrogen). Cycle sequencing reactions were performed by BigDye terminator v3.1 (3730 DNA analyzer; Applied Biosystem) by Giga Genomics Facility (Liège University, Belgium). DNA sequencing was performed in both directions. A consensus sequence was made according to the results of sequencing of the PCR products and the cloned PCR products via BioEdit program (Hall, 1999). The consensus sequences were analyzed

using BLASTn searches in GenBank (www.ncbi.nlm.nih.gov) and aligned using Clustal W software (Thompson et al., 1994) (sequence alignment is available from the authors upon request). Phylogenetic analysis was performed from consensus and reference sequences by using the neighbour-joining method with Mega 4 program (Tamura et al., 2007). The support for the tree nodes was calculated with 1000 bootstrap replicates. *Babesia* (*T.*) *microti*-positive samples were analyzed for potential co-infection with *Borrelia* spp. following a PCR protocol amplifying a part of the ospA gene (Demaerschalck et al., 1995). Plasmid containing genomic DNA of *Borrelia afzelii* (kindly supplied by Utrecht University) was used as positive control. Samples positive for *Borrelia* spp. were sequenced. Cycle sequencing reactions were performed using the Sanger method by the Medical Centrum Genetica (Ghent University Hospital, Belgium).

3. Results

Among the ticks collected from 561 dogs and 416 cats, four tick species were present: *Ixodes hexagonus*, *Ixodes ricinus*, *Dermacentor reticulatus*, and *Rhipicephalus sanguineus* (Table 4). Eight hundred forty-one out of the 1005 tick extracts were selected and validated by PCR amplifying the 16S rRNA tick gene. Of the 335 samples tested for inhibitors, a DNA dilution factor of 1/10 most adequately reduced the PCR inhibition. Without dilution, 85% of samples tested were inhibited. With a 1/10 dilution, 80% of the 335 tick extracts were positive. Engorgement had an effect. At a 1/10 dilution, 82% (n=55) of the negative samples were collected from engorged or semi-engorged ticks, whereas only 11% (n=32) of the positive samples were collected from engorged ticks. In some samples negative at a 1/10 dilution, increasing the dilution factor to 1/100 improved the sensitivity and some tested positive at a 1/1000 dilution (Figure 22).

The minimal DNA concentration allowing the detection of the 411-452 bp was estimated at 94 pg/μL (Figure 23). Eleven samples out of the 841 selected and validated DNA extracts were positive for *Babesia* spp; all were *I. ricinus* giving an infection rate estimated at 1.31% (95% CI: 0.65–2.33). These infected ticks had been collected from healthy cats and dogs, all living in rural areas and allowed to walk freely in gardens, wooded areas, and meadows. None of the other ticks from these same animals were positive. Positive samples were located throughout Belgium (Figure 24). In positive samples, PCR products were sequenced and results are available for seven. The 11 positive samples were also cloned: 4 samples could not

be cloned or sequenced, whereas 7 produced results (including 2 samples found negative through direct sequencing). Following the consensus sequences, three samples (accession no. GQ856649, GQ856652, and GQ856653) had 99% homology with *B.* (*T.*) *microti* (accession no. AF231349) and six others (accession no. GQ856650, GQ856651, GQ856654, GQ856655, GQ856656, and GQ856657) with *Babesia* sp. EU1 (accession no. AY046575). A single *B.* (*T.*) *microti*-positive sample (found in a nymph from Herentals) was also positive for *Borrelia burgdorferi sensu stricto.* A phylogenetic tree including each of the nine sequenced *Babesia* samples and some reference sequences is given in Figure 25.

	Adult		Nymph	Larva	Total
	Male	Female			
I. ricinus	22	747	7	0	776
I. hexagonus	1	152	47	1	201
D. reticulatus	3	21	0	0	24
R. sanguineus	0	4	0	0	4
Total	26	924	54	1	1005

Table 4: Ticks collected on dogs and cats during a one-year survey (April 2008-2009) in Belgium.

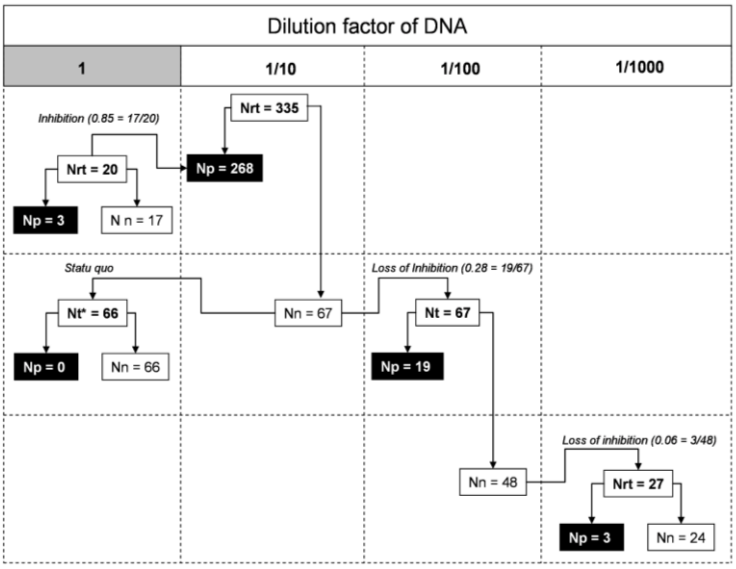

Figure 22: Influence of the DNA dilution factor on the 16S rRNA gene tick PCR inhibition.

(Nt: Number of samples tested; Nrt: Number of samples tested randomly; Np: Number of positive samples; Nn: Number of negative samples; * For one sample the test was not performed.)

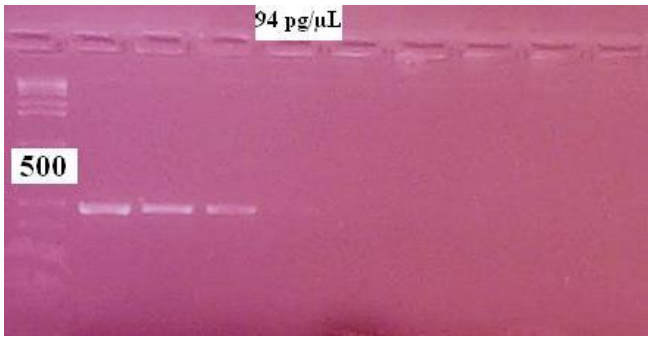

Figure 23: Sensitivity of the *Babesia* genus specific PCR was estimated at 94 pg/µL.

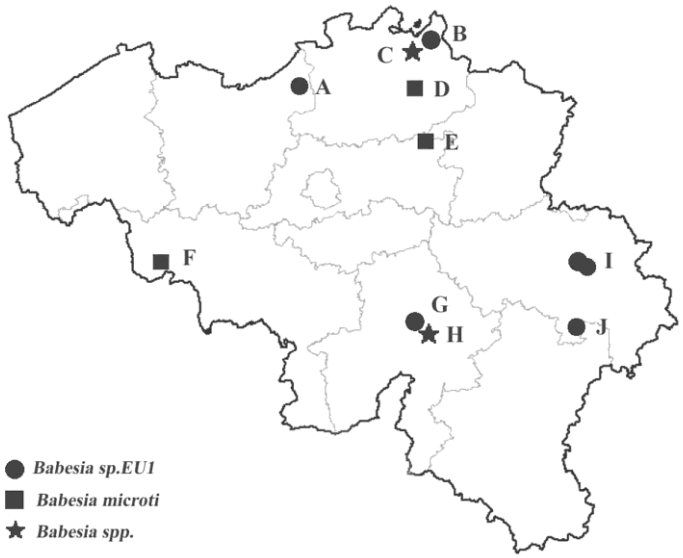

Figure 24: Map of Belgium showing the localities where *Babesia* spp. were found in *I. ricinus*.

(A: Beveren, B: Ravels, C: Beerse, D: Herentals, E: Zichem, F: Bruyelles, G: Spontin, H: Ciney, I: Theux, J: Arbrefontaine). Two *Babesia* sp. EU1 were found in Theux.

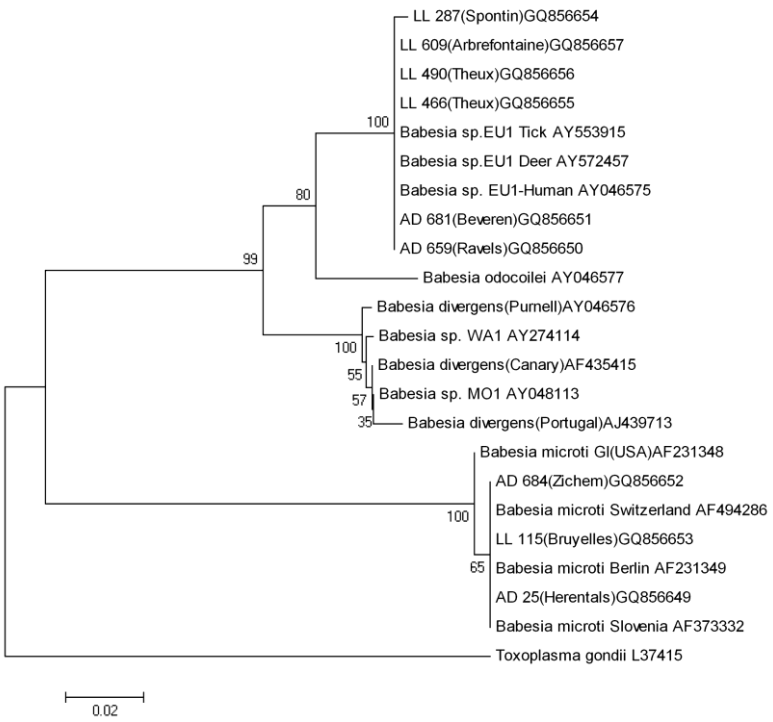

Figure 25: Phylogenetic tree based on a fraction (411-452bp) of 18S rRNA gene of selected *Babesia* spp.

The accession numbers for the reference sequences follow the corresponding names in the tree. Samples are identified with a code (2 letters and number); geographical locations are in brackets follow by the GenBank accession number.

4. Discussion

This study, based on molecular identification, shows an infection rate with *Babesia* spp. in *I. ricinus* in Belgium of 1.31% (95% CI: 0.65%–2.33%), which is in agreement with the data from nearby countries, that is, the Netherlands (0.6%–2.3%) (Nijhof et al., 2007; Wielinga et al., 2008), southern Germany (1%) (Hartelt et al., 2004), and Switzerland (0.7%–1.7%) (Casati et al., 2006). In central and eastern European countries, infection rates reach much higher values: 16.3% in Poland (Skotarczak and Cichocka, 2001), 9.6% in Slovenia (Duh et al., 2001) and up to 51% in Austria (Blaschitz et al., 2008). These marked differences could be due to environmental factors controlling tick density. Also, the selected collection technique (from an animal host or from the environment through flagging) could also influence detection rate. This study, based on the amplification of a fragment of the 18S

rRNA gene, has demonstrated, for the first time, *B.* (*T.*) *microti* and *Babesia* sp. EU1 in *I. ricinus* ticks collected from pets throughout Belgium. Both species were known to occur in several European countries (Casati et al., 2006; Bonnet et al., 2007; Wielinga et al., 2008). Taking into account the fact that other ticks collected from a pet carrying a positive tick were all negative, no conclusion can be drawn about transmission through co-feeding. The following questions remain: is *Babesia* sp. EU1 a new species? What are exactly the etiologic agents of human babesiosis in Europe?

Based on morphological criteria, serological cross-reactivity (Herwaldt et al., 2003), vector identity (Becker et al., 2009; Bonnet et al., 2009), and host range, *B. divergens* and *Babesia* sp. EU1 could belong to the same species. Until now, the main differences between *B. divergens* and *Babesia* sp. EU1 are based on a 31 bp difference on the 18S rRNA gene and the inability of *Babesia* sp. EU1 to infect and kill gerbils (*Meriones unguiculatus*) after experimental infection (Herwaldt et al., 2003). The phylogenetic tree (Figure 25) is in agreement with other authors' finding: *Babesia* sp. EU1 is close to *B. odocoilei* and is in a cluster group with *B. divergens* (Häselbarth et al., 2007). Unfortunately, in Europe, very few molecular data are available from confirmed human cases of babesiosis (Olmeda et al., 1997), and consequently, in many cases, the accurate identity of the responsible pathogen is unknown. It remains unclear why the epidemiology of *B.* (*T.*) *microti* seems different in the United States (where zoonotic impact is well known) and Europe (where confirmed human cases are very rare). Some hypotheses exist: these differences might be due to the circulation of different strains as suggested by the phylogenetic tree or due to the fact that others tick species are the vectors in the United States and Europe; and possibly, in Europe, the real prevalence in humans is underestimated because of subclinical infections (Persing et al., 1992) or co-infection with other tick-borne pathogens such as *Borrelia* spp. (Krause et al., 2002). This co-infection has again been demonstrated in the present animal survey. Further, the lack of specific diagnostic tools may explain the paucity of information about this group of pathogens in Europe. In this study, ticks collected from dogs and cats were examined. These two species live in close contact to humans and are exposed together with their owners, through travel and leisure activities, to *I. ricinus* ticks and the pathogens they carry. In this respect, pets could be considered as sentinels for tick-borne diseases such as borreliosis, anaplasmosis (Olson et al., 2000; Duncan et al., 2005), and babesiosis. This seems to be confirmed in this survey at least for the genus *Babesia*.

Several compounds in blood have been suggested to be PCR inhibitors, such as hemoglobin, lactoferrin, and immunoglobulin G. Hemoglobin and lactoferrin contain iron; the inhibitory

effects of both proteins may be related, in part, to their ability to release iron ions (Al Soud and Radstrom, 2001). When they feed, ticks ingest all of these components. Concerning our investigations, inhibition occurs in most cases when ticks were engorged. We assumed that these PCR inhibitions were probably due to these blood compounds.

B. Article 2: Longitudinal field study on bovine *Babesia* spp. and *Anaplasma phagocytophilum* infections during a grazing season in Belgium

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1. Introduction

Babesiosis and anaplasmosis are major tick-borne diseases with a high economic impact on livestock productivity over most of the world. Both diseases are also a public health concern and are caused by intraerythrocytic protozoa of the genus, *Babesia* spp. and bacteria within the genus *Anaplasma*, respectively. *Ixodes ricinus*, the most prevalent tick species in Northern and Western Europe, is an important vector of both these pathogens (Baumgarten et al., 1999; Becker et al., 2009; Bonnet et al., 2009).

In Europe, cattle are known to be the host of *Babesia divergens*, the aetiological agent of red water fever. *Babesia divergens* has also been found in humans and wild ruminants, roe (*Capreolus capreolus*) and red deer (*Cervus elaphus*) (Duh et al., 2005; Zintl et al., 2011), although the designation of red deer as a host is still under discussion. *Babesia divergens* is endemic in the southern part of Belgium but is considered absent from other parts of the country (Saegerman et al., 2007). To date, as far as we are aware, human babesiosis has never been reported in Belgium.

The availability of new molecular tools has considerably increased knowledge on the epidemiology and taxonomy of *Babesia* spp. A novel species recently described in Europe is *Babesia* sp. EU1, proposed as *B. venatorum* (Herwaldt et al., 2003). Roe deer are thought to be the natural host of this potentially zoonotic species. *Babesia* sp. EU1 is closely related to *Babesia odocoilei* (a parasite of white-tailed deer) and based on a 31 nucleotide difference over the complete 18S rRNA gene sequence has been placed phylogenetically in a sister

group with *B. divergens* (Herwaldt et al., 2003). Detection of *Babesia* sp. EU1 has been recorded recently in Belgium (Lempereur et al., 2011).

Anaplasma phagocytophilum is a polymorphic Gram negative bacterium that strictly parasitizes mammals; wild and domestic animals are the main reservoirs (Halos et al., 2010), but infection is potentially zoonotic causing Human Granulocytic Anaplasmosis (HGA). The symptoms of this disease vary from asymptomatic infection to severe disease by way of non-specific flu-like clinical signs (Bakken and Dumler, 2008; Thomas et al., 2009). *Anaplasma phagocytophilum* causes haematologic changes characterized by bacteriaemia, leucopenia and thrombocytopenia. The loss of white blood cells promotes suppression of the immune response and causes infected humans or animals to be more susceptible to other infectious pathogens (Woldehiwet, 2006).

In cattle, *A. phagocytophilum* causes Bovine Granulocytic Anaplasmosis (BGA), also called Tick-Borne Fever (TBF) (Woldehiwet, 2006). The symptoms associated with BGA are fever, a drop in milk production, joint swelling and abortion. In addition, many asymptomatic cases of infection are suspected (Pusterla and Braun, 1997). In Europe, the majority of clinical cases are observed in June and September, when temperature and humidity are optimal for ticks and cattle are grazing tick infested pasture (Pusterla et al., 1998). In Southern Belgium, the first case was reported in 2005 (Guyot et al., 2011). BGA is thus regarded as an emergent disease in Southern Belgium.

Because of numerous suspected cases of bovine anaplasmosis and the proximity of some cattle herds to wildlife habitats, this study was performed to measure seroprevalences for *A. phagocytophilum* and *Babesia* spp. in blood samples of cattle from farms in Southern Belgium. The prevalence of *A. phagocytophilum* and *Babesia* spp. infection was also assessed in both feeding and questing ticks collected from cattle and grazed pastures.

2. Materials and methods

Eight farms in Southern Belgium with a known history of anaplasmosis and/or babesiosis and where cattle are likely to have contact with wildlife were selected (Figure 26) (target sampling). Participation of the farms in the survey was voluntary.

a) Serological study

In each farm, 10 females (Holstein, Belgian White Blue or Montbéliard breed) older than one year of age were selected at random. Whole blood was taken into a dry container tube during spring (May-June), summer (July-August) and autumn (September) 2010. Centrifugation was performed at 3000 rpm for 5 minutes at room temperature and collected sera were stored at – 20 °C in 1 ml aliquots, until further use.

Sera were analyzed using an indirect immunofluorescence antibody test (IFAT) developed for *B. divergens* according to Gray and Kaye (1991). A *B. divergens* strain originating from a clinical case in Cherain (Province of Luxembourg, Belgium) was used as the antigenic source. A positive control serum was kindly provided by Pf. Pfister (Ludwig Maximilians University of Munich). Sera were tested at a dilution of 1:40 and 1:80.

Anaplasma phagocytophilum antibodies were also detected by IFAT, using an IFA detection kit (VMRD, Pullman, USA) at a 1:40 dilution.

b) Ticks collection and analysis

In the participating farms ticks were collected monthly, between May and September 2010, both from cattle (feeding ticks) and from the environment (questing ticks). In two farms the animals were grazing on two distinct areas (Figure 26). Ticks were trapped in grazed pastures by flagging with a 100 by 100 cm flannel cloth, performed when conditions were sufficiently dry (Bram, 1978). Farmers collected ticks from cattle using forceps, mainly during milking.

Ticks were preserved in ethanol 70 % and morphologically identified up to stage and species level using a standard key for morphological identification (Arthur, 1963). Sex and level of repletion were also recorded.

Tick DNA extraction was performed on adults and nymphs using proteinase K (20mg/mL) and the method of Boom et al. (1990). This method is based on lytic activity and nuclease inactivating properties of proteinase K together with the nucleic acid-binding properties of silica particles following by ethanol/acetone purification. To remove potential false-negative results due to Polymerase Chain Reaction (PCR) inhibition and to validate the efficiency of

the DNA extraction, an initial PCR test targeting the tick 16S rRNA gene was performed using 16S+1 and 16S-2 primers (Baumgarten et al., 1999). PCR negative samples were subsequently diluted to try to remove any potential inhibition.

A *Babesia* spp. genus-specific PCR based on the 18S rRNA gene was performed on validated DNA extracts, as described by Casati et al. (2006) and adapted by Lempereur et al. (2011). Tick DNA samples positive for *Babesia* spp. were purified using the Qiaquick PCR purification Kit (Qiagen, Germany). Cycle sequencing reactions were then performed in both directions, using a BigDye terminator v3.1 (3730 DNA analyzer; Applied Biosystems) by Giga Genomics Facility (Liège University, Belgium) and by DBS Genomics (Durham University, UK). A consensus sequence representing each PCR amplicon was generated and sequence comparison performed using BLASTn searches in GenBank (www.ncbi.nlm.nih.gov) and alignment with Clustal W software (Thompson et al., 1994).

Detection of *A. phagocytophilum* DNA was performed by quantitative Real-Time PCR (ABI 7000, Applied Biosystems) using Adiavet® Ana Pha Realtime kit (Adiagene, Saint Brieux, France). This diagnostic kit can detect 16S rRNA gene of 3 biovars of *A. phagocytophilum* (HGA, BGA and EGA).

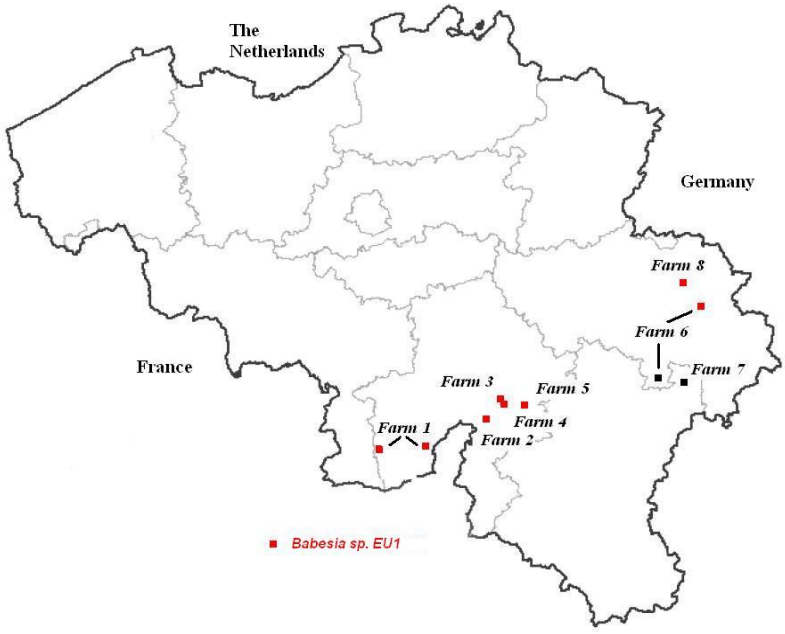

Figure 26: Map showing the selected farms. Farm 1 and Farm 6 have 2 different exploitation sites. In red, *Babesia* sp. EU1 found in ticks collected from cattle and grazed pastures.

(Farm 1: Aublain/Vierves-sur-Viroin; Farm 2: Finnevaux; Farm 3: Foy-Notre-Dame; Farm 4: Celles (Houyet); Farm 5: Chevetogne; Farm 6: Lierneux/Sart-lez-Spa; Farm 7: Vielsalm; Farm 8: Stembert)

3. Results

a) Serology

Blood samples were collected in spring, summer and autumn from 65 cows on 7 different farms (Table 5). Unfortunately, field conditions during the summer prevented the collection of blood samples from 15 animals of the initial group of 80 selected for sampling.

Babesia spp. seroprevalences of 10.7%, 20% and 12.3% were recorded in spring, summer and autumn, respectively. Only one farm had no *Babesia* sero-positive animals recorded throughout the experimental period. All farms had *A. phagocytophilum* positive animals. The seroprevalences were 30.8 %, 77 % and 56.9 % in spring, summer and autumn, respectively. Twelve (18.5%) and 17 (26.2%) cows were found to be either sero-negative or sero-positive throughout the complete period of the survey. 55.3% (36) of the animals presented a conversion in their serological status for *A. phagocytophilum*. Eleven cows (16.9%) presented 2 seroconversions during the study period of 5 months; recorded as positive from the spring and autumn samples but then negative in the summer, or only detected as sero-positive in sample collected in the summer. Seventeen animals were positive before turn out to pasture in the spring; all of them had been grazed the previous year. Seventeen cows were found to be seropositive for both *Babesia* spp. and *A. phagocytophilum* during the same period of sampling. Analysis of 2x2 contingency table (Table 6) gave a P value of 0.5440 using Fisher's exact test and consequently showed no statistically significant association between variables.

	Babesia spp.			*A. phagocytophilum*		
	Spring	Summer	Autumn	Spring	Summer	Autumn
Farm 1	2	7	0	8	9	9
Farm 2	0	0	2	1	6	3
Farm 3	0	0	0	0	5	0
Farm 4	2	0	1	5	6	3
Farm 5	0	6	5	2	4	4
Farm 6	2	0	0	1	10	10
Farm 8	1	0	0	3	10	8
Total	7	13	8	20	50	37
Percentage (%)	10.7	20	12.3	30.8	77	56.9

Table 5: Number of seropositive cows and seroprevalence for *Babesia* spp. and *A. phagocytophilum*.

Seroprevalence	*A. phagocytophilum* +	*A. phagocytophilum* -	Totals
Babesia spp. +	17	11	28
Babesia spp. -	90	77	167
Totals	107	88	195

Table 6: 2x2 contingency table containing seroprevalence for *Babesia* spp. and *A. phagocytophilum* of 65 cows during spring, summer and autumn in order to test their independence.

b) Ticks

A total of 805 ticks were collected. All were identified as *Ixodes ricinus*, both from cattle (n=180) and pasture (n=625), as shown in Table 7. The infection rate for *Babesia* spp. in PCR validated tick samples was estimated to be 14.6% and 7.9% in attached and questing ticks, respectively, without any obvious seasonal pattern. Fifty five positive samples were identified as *Babesia* sp. EU1 after sequencing (accession n° JF922092-JF922099), on the basis of demonstrating 98 to 100% identity with the GenBank reference sequence (accession n° AY046575). Two *Babesia* positive samples could not be identified to the species level. *Babesia* positive samples were found in all 7 of the sampled farms (Figure 26).

The overall tick infection rate for *A. phagocytophilum* was 8%, but strong differences were noticed according to the physiological status and stage of the ticks (feeding/questing; nymph/adult). Thus, 21.7% of the ticks collected from cattle were positive whereas only 3% of questing ticks gave a positive signal. However, almost all ticks collected from cattle were adults, whereas a majority of questing ticks were nymphs. Five female ticks collected from cattle were positive both for *A. phagocytophilum* and *Babesia* sp. EU1.

		Larvae	Nymphs	Adults	Screened	Validated	*A. phagocytophilum*		%	*Babesia* spp.		%
							Nymphs	Adults		Nymphs	Adults	
Cattle	May	0	3	96	99	84	0	17	20.2	0	6	7.1
	June	0	0	24	24	21	0	13	61.9	0	0	0
	July	0	1	9	10	10	0	0	0	0	0	0
	August	0	0	44	44	39	0	4	10.3	0	17	43.6
	September	0	1	2	3	3	0	0	0	0	0	0
	Total	**0**	**5**	**175**	**180**	**157**	**0**	**34**	**21.7**	**0**	**23**	**14.6**
Pasture	May	0	229	27	256	239	3	3	2.5	8	1	3.8
	June	77	157	1	158	151	3	0	2	5	0	3.3
	July	0	0	0	0	0	0	0	0	0	0	0
	August	56	42	1	43	25	4	0	16	10	1	44
	September	15	20	1	21	14	0	0	0	8	1	64.3
	Total	**147**	**448**	**30**	**478**	**429**	**10**	**3**	**3**	**31**	**3**	**7.9**
Total		**148**	**453**	**205**	**658**	**586**	**10**	**37**	**8**	**31**	**26**	**9.7**

Table 7: *Ixodes ricinus* collected between May and September 2010 from cattle and on grazed pastures.

Nymph and adult ticks were validated and screened for *A. phagocytophilum* and *Babesia* spp. specific genomic DNA material.

4. Discussion

Infection of cattle with the tick-borne pathogens, *Anaplasma* and *Babesia*, can have a serious impact on productivity, with the level of disease threat within a particular geographical region being strongly influenced by the species of these pathogens predominantly transmitted by ticks. It is relevant, therefore, to have up to date information on the level of infection in cattle and the species of pathogens that are most likely to be transmitted by ticks within regions under threat of disease. In the current study the serological survey for *Babesia* spp. gave a prevalence (14.3 %) higher than the prevalence of 7.9% found in the same area by Losson et al. (1989). The seroprevalence to *A. phagocytophilum* was also higher when compared to other previous European publications (Amusategui et al., 2006; Ebani et al., 2008). The fact that all the participating farms participated on voluntary basis, had a confirmed history of tick-borne disease and were located in a known endemic area could explain the high seroprevalences, due to targeted sampling.

The high seroprevalence against *A. phagocytophilum* was detected despite evidence that antibodies titre rapidly wanes, as indicated by double sero-conversion results. This finding clearly has implications for detection of the true level of pathogen challenge that cattle encounter, and for both diagnostic and epidemiological studies, the timing of sampling is of critical importance and should be performed close to the peak period of tick activity. Nevertheless, a significant proportion (26%) of cattle were still positive before turn out to pasture suggesting that some cows remain as carriers throughout winter and may provide an overwintering reservoir population of *A. phagocytophilum* for feeding ticks in the spring.

BGA seems to be endemic in Southern Belgium, as in a recent survey of 1197 cattle, 320 tested positive for antibodies against *A. phagocytophilum* (ARSIA, unpublished data). This high level of infection and associated immunity could contribute to the relative lack of acute clinical cases of BGA in monitored herds. However, this may also be linked to *A. phagocytophilum* strain heterogeneity and vertebrate diversity (de la Fuente et al., 2005b; Granquist et al., 2010). Infection rates estimated for *Babesia* spp. in ticks were higher than generally expected for this region (Lempereur et al., 2011). This could well be due to targeted sampling, as mentioned above. However the high density of cervid populations in the study area may also have played a role. The infection rate found in ticks collected from cattle was also higher than that estimated for unfed questing ticks. It should be borne in mind that the

ticks collected from cattle were all adult ticks while the majority of those collected from the vegetation were nymphs. While the infection rates of both adult and nymph ticks will be influenced by the level of *Babesia* infection in the mammalian host, the known amplification of parasite in the tick salivary gland following a blood meal may increase the chances of detection by the PCR test. Furthermore, the adult population will be comprised of ticks that are transmitting and acquiring infection from a definitive host for *Babesia*, while the infection level in the nymph population will be predominately derived from adult female ticks (Zintl et al., 2003) that could, theoretically, have fed on a range of vertebrate hosts.

Babesia sp. EU1 was the only species found in both feeding and questing *I. ricinus*, despite the high number of ticks that were screened and the selection of farms located in a region previously identified as endemic for *B. divergens*. The same finding was previously described by Becker et al. (2009), who found no evidence for *B. divergens* sporozoites in engorged adult or unfed *Ixodes ricinus* ticks. Cattle and deer are the main hosts of adult *Ixodes ricinus* ticks and are potential carriers of *B. divergens*. One possible explanation for our results could be that the main stage of *I. ricinus* transmitting *B. divergens* in the study area is the larvae after transovarial transmission. This possibility would need further investigation, as the accepted major transmission route is via feeding adults following transovarian transmission.

As deer can probably be a reservoir host for *B. divergens* (Zintl et al., 2011), it may be that cattle play the same role for *Babesia* sp. EU1 in natural habitats characterized by deciduous forests with large populations of wild ruminants. This hypothesis has yet to be proven, indeed experimental validation of cattle acting as a reservoir for a *Babesia* species of deer has only been tested on *B. capreoli* (Adam et al., 1976; Gray et al., 1990; Malandrin et al., 2010). If it can be validated that cattle can act as a reservoir for *Babesia* sp. EU1 it would raise implications for the epidemiology and control of this zoonotic parasite.

Wild cervids appear to act as a reservoir host for *A. phagocytophilum* (Rosef et al., 2009) as do probably, cattle (de la Fuente et al., 2005a; Halos et al., 2010).

Since 1975, wild ruminant populations have doubled in Southern Belgium, despite an increase in hunting activity (Libois, 2006; SPW - DGO3 Agriculture-Ressources naturelles et Environnement, 2011). Expanding populations of wild cervids that act as important hosts for maintaining tick populations and acting as a reservoir for zoonotic tick-borne pathogens have the potential to alter the balance between the parasite and additional, potentially human, hosts. Therefore, knowledge of common animal reservoirs for transmission of zoonotic *Babesia* sp.

will be useful for modelling the risk potential of this infection in humans: the results of this study implicate cattle as well as cervids.

The high seroprevalence to *A. phagocytophilum* in cattle suggest this pathogen is endemic in Southern Belgium. It would be expected, therefore, that ticks and mammalian hosts would be co-infected with *A. phagocytophilum* and additional tick-borne pathogens. Nevertheless, despite evidence of co-infection in 17 cattle and 5 ticks, no statistically significant evidence of relation was shown between seroprevalence to *Babesia* spp. and *A. phagocytophilum* in this study (Table 6). It is known that co-infection of livestock, synchronous or not, with tick-borne disease (TBD) can exacerbate disease morbidity and reduce host fitness (Zintl et al., 2003). Furthermore recent studies have provided evidence that interaction between tick-borne pathogen populations can influence TBD epidemiology. This can be manifest in a positive or negative manner and alter pathogen prevalence, pathogen transmission and host susceptibility (Ginsberg, 2008; Telfer et al., 2010). Therefore, to gain fuller insight into the factors that influence risk from tick-borne disease, future studies should attempt to capture data on interacting parasite communities rather than individual parasite species.

C. Article 3: Wild cervids are host for tick vectors of *Babesia* species with zoonotic capability in Belgium

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Key Words: *Babesia* species, Belgium, Cervids, Tick, Zoonotic.

1. Introduction

Babesiosis is a tick-borne disease caused by different species of intraerythrocytic protozoa classified within the genus *Babesia*. Different species of *Babesia* are described as potentially zoonotic and cause a malaria-like disease in humans. Interest in *Babesia* species with zoonotic capability has increased recently and babesiosis has been described by some authors as an emergent zoonotic disease (Hunfeld and Brade, 2004; Vannier and Krause, 2009; Gray et al., 2010). Indeed, since the first case in 1957, the number of identified human babesiosis cases have increased steadily, mostly in the USA but also in Europe, and while the majority of cases reported in Europe were in immuno-compromised individuals, cases have been reported also in non asplenic patients (Martinot et al., 2011).

A new species of *Babesia* has recently been described in Europe: *Babesia* sp. EU1, proposed as *B. venatorum,* is a new candidate species and was identified for the first time in two asplenic patients (Herwaldt et al., 2003). *Babesia* sp. EU1 has also been identified in roe deer (*Capreolus capreolus*), (Duh et al., 2005; Bonnet et al., 2007) and *B. divergens* and *B. capreoli* have been described as occurring in wild European cervids (Malandrin et al., 2010; Zintl et al., 2011). The clinical symptoms if rarely generated by these parasites in wild cervids appear to be the same as those described in their domestic counterparts but have been documented less frequently (Penzhorn, 2006). Characterisation of these species has essentially been based on a comparison of the complete sequence of the 18S rRNA gene. *Babesia* sp. EU1 seems to be closely related to *Babesia odocoilei* (a parasite of white-tailed

deer) with divergence of 28 nucleotides (Herwaldt et al., unpublished data), and phylogenetically is placed in a sister group with *B. divergens*, with 31 nucleotides of difference (Herwaldt et al., 2003). *Babesia capreoli* has recently been redescribed and seems to only display 3 nucleotides difference over the 18S rRNA gene, relative to *B. divergens* (Malandrin et al., 2010). Vectorial competence of *Ixodes ricinus*, the most common tick species in Europe, has been proven for all 3 of these *Babesia* species (Nikol'skii and Pozov, 1972; Donnelly and Peirce, 1975; Becker et al., 2009; Bonnet et al., 2009). Numerous reasons for the apparent recent emergence of zoonotic *Babesia* species have been suggested. These include changes in human behaviour, activities that generate closer proximity to tick habitats and a more systematic approach to diagnosis, with awareness of the possibility of co-morbidity with *Borrelia* spp. or subclinical infection. Expanding populations of wild cervids that act as important hosts maintaining tick populations and acting as reservoir hosts for some zoonotic tick-borne pathogens is also suspected.

Because of the recent first report of potentially zoonotic *Babesia* species in the tick *Ixodes ricinus* in Belgium (Lempereur et al., 2011), combined with sparse information regarding the prevalence of the different *Babesia* species and their potential zoonotic impact, this study was performed to investigate the range of *Babesia* species found in ticks collected from wild cervids in Belgium.

2. Materials and Methods

In 2008, visible ticks were systematically collected from wild cervids (*Cervus elaphus* and *Capreolus capreolus*) found dead, hunted or killed for sanitary reasons in the context of the disease monitoring activities of the WildScreen Network in Southern Belgium (Linden et al., 2011). Ticks were preserved in ethanol 70 % and morphologically identified up to stage and species level using a standard key for morphological identification (Arthur, 1963). Sex and repletion level were also recorded. Ticks were frozen in liquid nitrogen and immediately homogenized in a Tissue Lyser® (Qiagen, Germany). DNA extraction was performed for individual ticks using a NucleoSpin tissue kit (Macherey-Nagel GmbH, Germany). DNA of males was extracted with the XS version of the kit.
DNA quantity and quality was evaluated for each extracted sample using a spectrophotometer (Nanodrop, Thermo scientific). To remove potential false-negative results due to polymerase

chain reaction (PCR) inhibition and to validate the efficiency of the DNA extraction, an initial PCR test targeting the tick 16S rRNA gene was performed, using primers TQ16S+1F (5'-CTG CTC AAT GAT TTT TTA AAT TGC TGT GG-3') and TQ16S-2R (5'-ACG CTG TTA TCC CTA GAG-3') (Black and Piesman, 1994; Halos et al., 2004). The *Babesia* spp. genus-specific PCR based on the amplification of a 411–452-bp fragment located at position 488-912 of the 18S rRNA gene and using BJ1 (5'-GTC TTG TAA TTG GAA TGA TGG-3') and BN2 (5'-TAG TTT ATG GTT AGG ACT ACG-3') primers was then applied on validated DNA extracts (Casati et al., 2006; Lempereur et al., 2011).

Babesia spp. positive amplicons generated from the tick DNA samples were sequenced following purification with the Qiaquick PCR purification Kit (Qiagen, Germany). Cycle sequencing reactions were performed in both directions by BigDye terminator v3.1 (3730 DNA analyzer; Applied Biosystems) by Giga Genomics Facility (Liège University, Belgium) and by DBS Genomics (Durham University, UK). A consensus sequence representing each PCR amplicon was generated and sequence comparison performed using BLASTn searches in GenBank (www.ncbi.nlm.nih.gov) and alignment with ClustalW software (Thompson et al., 1994).

3. Results

A total of 1044 ticks were collected from 47 cervids (34 *Cervus elaphus* and 13 *Capreolus capreolus*) found dead, hunted or killed for sanitary reasons in 41 locations situated in 4 different provinces (Liège, Luxembourg, Namur, Brabant Wallon) (Figure 27). The number of ticks per animal ranged between 1 and 89. These ticks were all identified as *Ixodes ricinus* (23 nymphs, 678 females and 343 males). From these ticks, 1023 DNA extracts were validated and subsequently tested by PCR for the presence of *Babesia* spp. DNA.

From these extracts twenty eight generated *Babesia* PCR amplicons and were designated as positive, giving a tick infection rate of 2.7% (95% CI: 1.8 – 3.9%). The positives results were found in a nymph (1) or adult ticks, females (15) and males (12), collected from 12 different cervid hosts (7 roe deer and 5 red deer). From analysis of the sequences derived from the *Babesia* positive amplicons, three samples [GenBank: JF776397-JF776398, JF776408] were identified to contain *B. divergens* with 100% of similarity to the GenBank sequence

[AY046576], five samples [GenBank: JF776393, JF776399, JF776400-JF776402] showed 98 and 99 % of homology and were designed as *B. divergens*-like (Table 8). Eleven samples [GenBank: JF776394, JF776406, JF776407, JF776410-JF776417] were identified as *Babesia* sp. EU1 after demonstrating 100 % of similarity with the reference sequence [GenBank: AY046575]. Three samples [GenBank: JF776395, JF776396, JF776405] were identified as *Babesia* sp. EU1-like. *Babesia divergens* and *Babesia* sp. EU1 DNA was identified in extracts of ticks collected from both roe and red deer. Two *Babesia* amplicons generated from independent tick extracts [GenBank: JF776403, JF776404] show 100% sequence identity with the *B. capreoli* reference sequence [GenBank: FJ944828]. Both ticks were collected from the same roe deer in the province of Liège. *Babesia* sequences derived from four tick extracts could not be identified at the species level and were identified as *Babesia* sp. [GenBank: JF776409] (see Table 8). The geographical distribution of the validated and the positive samples for *Babesia* spp. DNA, found in the province of Liège, Luxembourg and Namur is shown in Figure 27.

Figure 27: Map of Belgium showing 41 localities situated in 4 different provinces (Liège, Luxembourg, Namur and Brabant Wallon), from where the 47 cervids originated.

Babesia positive samples collected from 12 different deer are localised on the map. One *Babesia*-positive tick sample was collected from a deer with unknown origin. In two localities (La Reid and Briquemont), more than one *Babesia* spp. were found in different ticks collected from the same deer. Negative samples were designated by X.

Babesia species	Genbank n°	Modification at nucleotide position respecting to *B. divergens* (AY046576)																																															
		500	501	503	504	506		600	602	603	609	612	615	616	617	618	619	620	621	622	626	630	631	632	633	637	638	639	640	641	643	644	645	646	663	768	804	808	843	851	852		853	878	886	890	902	907	908	909	911
B. divergens	**AY046576**	T	C	C	C	G		G	T	G	T	C	G	G	T	G	T	T	A	A	T	T	A	A	T	G	A	G	A	T	G	C	A	C	A	G	G	T	T	A	C		G	G	T	T	A	T	A	G	G
JF776397; JF776398;	JF776408*	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-
B. divergens-like	JF776393	A	A	A	A	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	/	-	-	-	-		-	-	-	-	-	-	-	-	-
	JF776399	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		-	-	-	/	-	-	-	-	-
	JF776400	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-		A	A	-	-	T	A	G	T	C
	JF776401	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		-	-	-	/	-	/	/	A	-
	JF776402	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		-	-	-	/	-	-	-	-	-
Babesia sp. EU1	**AY046575**	-	-	-	-	-		-	-	-	C	-	T	A	-	C	G	A	G	T	-	-	C	T	-	T	T	T	-	A	C	G	-	T	-	T	-	-	-	-	-		-	-	C	-	-	-	-	-	-
JF776394; JF776406;	JF776407*	-	-	-	-	-		-	-	-	C	-	T	A	-	C	G	A	G	T	-	-	C	T	-	T	T	T	-	A	C	G	-	T	-	T	-	-	-	-	-		-	-	-	-	-	-	-	-	-
	JF776410-JF776417*	-	-	-	-	-		-	-	-	C	-	T	A	-	C	G	A	G	T	-	-	C	T	-	T	T	T	-	A	C	G	-	T	-	T	-	-	-	-	-		-	-	-	-	-	-	-	-	-
Babesia sp. EU1-like	JF776395	-	-	-	-	-		-	-	-	C	-	T	A	-	C	G	A	G	T	-	-	C	T	-	T	T	T	-	A	C	G	-	T	-	T	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-
	JF776396	-	-	-	-	-		-	-	-	C	-	T	A	-	C	G	A	G	T	-	-	C	T	-	T	T	T	-	A	C	G	-	T	-	T	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-
	JF776405	-	-	-	-	-		-	-	-	C	-	T	A	-	C	G	A	G	T	-	-	C	T	-	T	T	T	-	A	C	G	-	T	-	T	-	/	/	-	-		-	-	-	-	-	-	-	-	-
B. capreoli	**FJ944828**	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-
JF776403;	JF776404*	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-
Babesia sp.	JF776409	-	-	-	-	-		T	G	/	-	T	T	A	A	A	A	G	-	G	C	A	T	T	G	-	T	T	T	A	C	G	G	T	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-

Table 8: Differences in the 18S rRNA gene partial sequence of the *Babesia* positive samples respecting to *Babesia divergens* (AY046576). / show deletions. Addition of nucleotide was shown by an additional column following the nucleotide position. * show samples with 100 % of similarity with the species reference.

4. Discussion

This study confirms the presence of *B. capreoli* together with two potentially zoonotic *Babesia* species in Belgium, most notably *Babesia* sp. EU1, in ticks collected from wild ruminants. The estimated *Babesia* spp. infection rate (2.7%) is in agreement with other previous publications (Iori et al., 2010; Lempereur et al., 2011).

Two samples were identified as *B. capreoli* in the province of Liège. The differentiation between *B. capreoli* and *B. divergens* is difficult due to their morphological similarities and strong serological cross reactivity and it is also complicated due to the high percentage of identity between the sequences of their respective 18S rRNA genes. In this survey, species identification was based on the difference of 2 nucleotide bases on the amplified fragment of 18S rRNA gene at position 631 and 663, following the recent redescription of *B. capreoli* by Malandrin and associates (2010) (see Table 8). Although *B. capreoli* is not considered to be a zoonotic species of *Babesia*, this parasite can infect various members of the cervidae family (Adam et al., 1976; Gray et al., 1990) and also alpine chamois (Hoby et al., 2009), but the roe deer seems to be most susceptible to infection (Malandrin et al., 2010). Based on the results of our sequence analysis we conclude that *B. capreoli* is present in Belgium, as suggested by the results of a previous serological study (Lonneux et al., 1991).

Identification of the potentially zoonotic *Babesia* sp. EU1 in ticks collected in Belgium in this study validated previous findings of Lempereur et al. (2011). The finding that one adult male tick collected from red deer was positive for *Babesia* sp. EU1 might be considered surprising, as *Babesia* sp. EU1 has been identified in human and in roe deer but never in red deer (Duh et al., 2005; Tampieri et al., 2008; Zintl et al., 2011). While it is possible that this result does reflect a genuine infection of red deer with this parasite, it is also possible, due to the propensity of adult male *I. ricinus* to feed often and intermittently and to transovarial transmission, that the positive identification represents a previous acquisition from a different host species. Further work analysing blood samples from the deer population would be required to support conclusion that the red deer is a reservoir host for *Babesia* sp. EU1.

Babesia sp. EU1 and *B. capreoli* are known to have cervids as host (Duh et al., 2005; Zintl et al., 2011), but the role of deer as reservoir for *B. divergens* is still controversial. In the genus *Babesia*, *B. divergens* has one of the largest ranges of host species, and is considered to be the main causal agent of human babesiosis in Europe. *Babesia divergens* has been found in roe

and red deer (Duh et al., 2005; Garcia-Sanmartin et al., 2007; Zintl et al., 2011), however, since it is difficult to distinguish this species from *B. capreoli*, misidentification is possible and further molecular based studies with a high level of species-specific fidelity are required. In cattle, this species is endemic in some areas of Southern Belgium and its distribution seems to be spreading (Losson, 1989; Losson and Lefèvre, 1989; Everaert et al., 2007). *Ixodes ricinus* is also the vector of other human and animal pathogens such as *Borrelia burgdorferi sensu lato* and *Anaplasma phagocytophilum*. While small rodents act as competent reservoirs for *B. burgdorferi*, wild cervids may play a similar role for *A. phagocytophilum*. Co-infections with *Babesia* spp. are probably fairly common and could enhance the severity of these protozoal infections in humans and animals (Welc-Faleciak et al., 2010; Hildebrandt et al., 2011b). Within Europe it is generally accepted that the risk of tick-borne diseases has increased and has potential to become of greater significance due to climate change and additional factors, such as altered human recreational activity and an increasing number of immuno-compromised patients. An important factor that could increase the risk of infection with zoonotic *Babesia* species is the expansion of reservoir host populations, as these promote maintenance of the parasite and allow transmission to the next generation of feeding ticks. Since 1975, wild ruminant populations have doubled in Southern Belgium, despite an increase in hunting activity (Libois, 2006; SPW - DGO3 Agriculture-Ressources naturelles et Environnement, 2011). Such a situation has the potential to alter the balance between the parasite and its additional, potentially human, hosts. Therefore, knowledge of the most common reservoir source for transmission of zoonotic *Babesia* spp. will be useful for modelling the risk potential of this infection to humans.

IV. Part II

A. Article 4: Development and validation of a PCR–RFLP test to identify African *Rhipicephalus* (*Boophilus*) ticks

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Key Words: *Rhipicephalus* (*Boophilus*), Identification, PCR-RFLP, Second internal transcribed spacer (ITS2), Africa.

1. Introduction

Ticks of the genus *Boophilus*, recently grouped as a subgenus of *Rhipicephalus*, are some of the most important tick species in the world from an economical point of view (Estrada-Peña et al., 2006). Five species compose this genus: *R.* (*Boophilus*) *annulatus*, *R.* (*Boophilus*) *decoloratus*, *R.* (*Boophilus*) *geigyi*, *R.* (*Boophilus*) *kohlsi* and *R.* (*Boophilus*) *microplus*. *Rhipicephalus* (*Boophilus*) *kohlsi* was not included in this study, as it is only present in the Middle East and found exclusively on sheep and goat. The remaining *Boophilus* species all colonize Africa and *R.* (*Boophilus*) *microplus*, being the most important cattle tick, has only just recently been introduced in West Africa (Madder et al., 2007). The recognition of this species amongst the three other closely related Boophilids, endemic in the area, is extremely difficult and could therefore easily be overlooked. The first indication of the introduction of this species was the failure of acaricide treatment as a result of the known high degree of resistance which characterizes this species (George et al., 2004). In all of the tropical and subtropical areas where *R.* (*Boophilus*) *microplus* had been introduced, the tick became a serious hindrance to livestock production. Parasitism by *R.* (*Boophilus*) *microplus* results in poor condition, weight loss, reduced meat and milk production, and potential transmission of

Babesia bovis, *B. bigemina* and *Anaplasma marginale* (Estrada-Peña and Venzal, 2006). Recent publications indicate an extension of the distribution of *R.* (*Boophilus*) *microplus* and the displacement of *R.* (*Boophilus*) *decoloratus* in different countries like in Tanzania (Lynen et al., 2008) and in South Africa (Tonnesen et al., 2004). The conventional method to identify *R.* (*Boophilus*) spp. relies on comparison of morphological characteristics of the different species, which is extremely cumbersome as size and differences between species are limited and sometimes even variable. To be able to identify *R.* (*Boophilus*) *microplus* with certainty and so differentiate the four cattle related *R.* (*Boophilus*) spp., the development of a PCR–RFLP, based on sequence differences in the second internal transcribed spacer (ITS2), was the main objective of this study. To validate the test, tick samples from West Africa were collected and identified both morphologically and genetically.

2. Materials and Methods

Sources of ticks

A preliminary cross-sectional tick survey was carried out in West Africa between May 2006 and February 2008 which yielded tick samples from nine countries: Senegal, Togo, Burkina Faso, Guinea-Conakry, Cameroon, Mauritania, Niger, The Gambia and Ivory Coast. In each country between 3 and 10 cattle were sampled. These ticks were stored in 70% alcohol, labeled and sent to the Institute of Tropical Medicine in Antwerp (Belgium) for analysis.

Morphological identification

The first step of the morphological identification of the ticks up the genus level was done using a stereomicroscope (Zeiss Stemi 2000) at 60× magnification. In a next step a microscope (Olympus) at the 100× magnification was used to identify *R.* (*Boophilus*) ticks at species level. For the latter, the hypostome dentition, presence of a protuberance bearing setae on palpal segment 1, external spur on coxae II and III, caudal process on male engorged ticks and shape of the internal and external spurs on adanal plates were used as discriminating characteristics (Walker et al., 2003). The morphological identification of a subset of the ticks was confirmed by Pf. Ivan Horak (Faculty of Veterinary Tropical Diseases, University of Pretoria, South Africa).

Development of PCR and RFLP technique

To develop the PCR and RFLP technique, 27 reference ticks were included: 9 *R.* (*Boophilus*) *microplus* adults from Ivory Coast, 8 *R.* (*Boophilus*) *decoloratus* of which 3 came from Cameroon, 2 from Ivory Coast and 3 from The Gambia. Were also included 5 *R.* (*Boophilus*) *geigyi*: 2 from Ivory Coast and 3 from Guinea as well as 5 *R.* (*Boophilus*) *annulatus* from Cameroon. The ITS2 region of some morphologically identified specimens was sequenced to confirm the initial identification.

Primers design

ITS2 sequences for *R.* (*Boophilus*) *microplus, geigyi, annulatus* and *decoloratus* (GenBank accession nos. U97715, AF271273, AF271272 and U97716) were downloaded and aligned using Clustal W software (Thompson et al., 1994). Primers were designed using a DNA computer software program Web primer (http://seq.yeastgenome.org/cgi-bin/web-primer). Amplification was done on the ITS2 gene using the forward primer Boophits2 F 5'-GCC-GTC-GAC-TCG-TTT-TGA-3' and Boophits2 R 5'-TCCGAA- CAG-TTG-CGT-GAT-AAA-3' as reverse primer. GC content and self-annealing was checked on the Oligo Calc web site (http://www.basic.northwestern.edu/biotools/oligocalc.html). These primers were tested in the AmpliX program (http://ifrjr.nord.univ-mrs.fr/AmplifX) and the expected amplicon lengths were estimated. For *R.* (*Boophilus*) *microplus* the expected length was 829 bp, for *R.* (*Boophilus*) *geigyi* 765 bp, for *R.* (*Boophilus*) *annulatus* 832 bp and for *R.* (*Boophilus*) *decoloratus* 821 bp.

DNA extraction

DNA was extracted using the method of Boom et al. (1990). This method is based on lytic activity and nuclease inactivating properties of proteinase K together with the nucleic acid-binding properties of silica particles following by ethanol/acetone purification.

DNA amplification

Standard PCR amplifications were carried out in 25µl reaction mixtures containing 5µl of the extracted DNA, 1.65mM $MgCl_2$, 0.2mMof the four dNTPs, 10pM of each primer, 1UTaq polymerase enzyme (Promega) and 1µl Yellow SubTM (GENEO Bioproducts, Hamburg, Germany). The reaction mixture was overlaid by a drop of fine neutral mineral oil (ICN) and placed on a heating block of a programmable thermocycler (Biometra, Westburg). After a denaturation step of 4min at 94 ◦C each of the 40 cycles consisted of 30 s at 92 ◦C, 45 s at 58 ◦C and 60 s at 72 ◦C and ended with an extension step of 8min at 72 ◦C. The mixtures were examined for the presence of DNAfragments by loading 5µl of each reaction mixed with 2µl of loading buffer onto 2% agarose gels (Sigma). A 1.5 kb DNA ladder (MBI Fermentas, Lithuania) was loaded on every gel. The samples were run for 20 min at 100 V, stained in ethidium bromide for 30 min, washed under running tap water and photographed under UV illumination. For further typing of the fragments, RFLP based methods were used.

Restriction fragment length polymorphism (RFLP)

Suitable restriction enzymes were identified with the aid of the online tools of The Restriction Enzyme Database (Rebase® http://rebase.neb.com/rebase/rebase.html). Restriction site positions and fragment lengths were determined with DNALC Sequence utilities (http://www.dnalc.org/bioinformatics/dnalc_nucleotide_analyzer.htm). PCR products were digested by Msp1 enzyme (6U) with 4µl of amplified DNA in 15µl total volume. The reaction was left overnight at 37 ◦C. Four microliters of all restricted sample mixed with 2µl of loading buffer was transferred onto a polyacrylamide gel and a 1 kb DNA superladder (Eurogentec) was added to all gels for fragment size determination. DNA fragments were separated by vertical electrophoresis in TBE buffer at 100V for 2.5 h. The gel was stained using Sybr Green I (Cambrex Bio Science, Rockland, Inc.) during 40 min.
Restriction sites are located for *R.* (*Boophilus*) *microplus* at 241 bp, 311 bp and 408 bp, for *R.* (*Boophilus*) *geigyi* at 108 bp, 323 bp, 333 bp, 385 bp, 709 bp, for *R.* (*Boophilus*) *annulatus* at 108 bp, 241 bp, 311 bp, 408 bp and for *R.* (*Boophilus*) *decoloratus* at 107 bp, 241 bp, 311 bp, 391 bp, 407 bp, 442 bp, 765 bp. Thus, in agarose gel, theoretically for *R.* (*Boophilus*) *microplus* four bands of 421 bp, 241 bp, 97 bp and 70 bp had to be found. For *R.* (*Boophilus*) *geigyi*, six bands of 324 bp, 215 bp, 108 bp, 56 bp, 52 bp and 10 bp. For *R.* (*Boophilus*)

annulatus five bands of 424 bp, 133 bp, 108 bp, 97 bp and 70 bp. For *R.* (*Boophilus*) *decoloratus* eight bands of 323 bp, 134 bp, 107 bp, 80 bp, 70 bp, 56 bp, 35 bp, 16 bp.

3. Results

The morphological identification of 1070 ticks revealed that the *R.* (*Boophilus*) specimens represented 39% of the collected ticks, predominantly *R.* (*Boophilus*) *geigyi* except in Ivory Coast where *R.* (*Boophilus*) *microplus* probably overtop all other *R.* (*Boophilus*) spp. One third of these *Boophilus* ticks remained unidentified at species level because of damaged mouthparts. The 27 reference ticks allowed the development of a PCR–RFLP. This PCR–RFLP allowed then the identification of the damaged *Rhipicephalus* (*Boophilus*) ticks.

The PCR amplification gave for 55 tested samples a major product between 765 bp and 832 bp (Figure 28) as predicted by AmpliX program. The RFLP using Msp I digestion gave very distinct profiles differentiating the four *Rhipicephalus* (*Boophilus*) species as theoretically expected (Figure 29). The profile of *R.* (*Boophilus*) *annulatus* showed five bands on agarose gel (around 400 bp, 150 bp, 100 bp, 90 bp, 75 bp) (Figure 29 columns 10–11), whereas *R.* (*Boophilus*) *microplus* showed four bands (around 400 bp, 250 bp, 100 bp, 70 bp) (Figure 29 columns 1–5). *Rhipicephalus* (*Boophilus*) *geigyi* was characterized by 5 bands (around 300 bp, 200 bp, 100 bp, and 2 bands around 50 bp). One band of 10 bp is missing due to the very short length of this fragment (Figure 29 column 6). *Rhipicephalus* (*Boophilus*) *decoloratus* showed 7 different bands (around 300 bp, 150 bp, 100 bp, 80 bp, 70 bp, 50 bp, and 30 bp). One band of 16 bp was missing also due to the very short length of this fragment (Figure 29 columns 7–9).

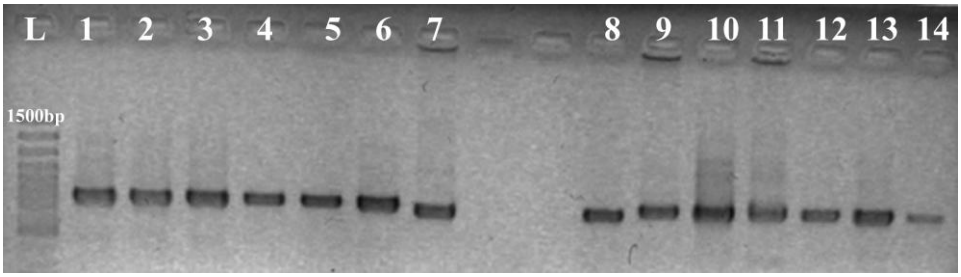

Figure 28: *Rhipicephalus* (*Boophilus*) ticks PCR products.

Ladder at the left (L), *R.* (*Boophilus*) *microplus* reference ticks in the columns 1–6, *R.* (*Boophilus*) *geigyi* reference ticks in column 7, *R.* (*Boophilus*) *decoloratus* reference ticks in columns 8–10 and *R.* (*Boophilus*) *annulatus* reference ticks in columns 11–13, positive control in column 14.

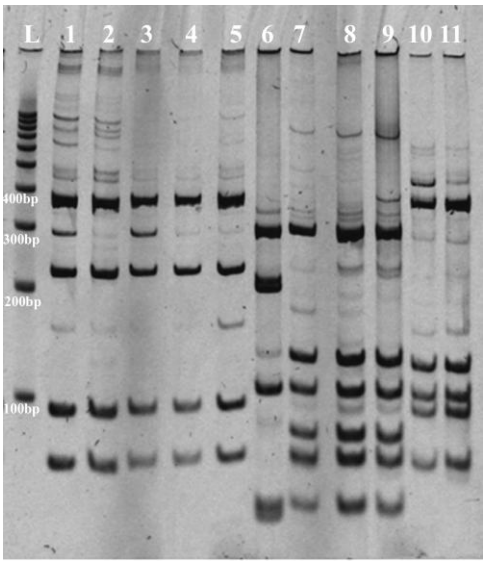

Figure 29: *Rhipicephalus* (*Boophilus*) ticks RFLP profiles.

Ladder at the left (L), *R.* (*B.*) *microplus* (bands around 400 bp, 250 bp, 100 bp, 70 bp) in columns 1–5; *R.* (*B.*) *geigyi* (bands around 300 bp, 200 bp, 100 bp, and 2 bands around 50 bp) in the column 6; *R.* (*B.*) *decoloratus* (bands around 300 bp, 150 bp, 100 bp, 80 bp, 70 bp, 50 bp, 20 bp) in columns 7–9; *R.* (*B.*) *annulatus* (bands around 400 bp, 150 bp, 100 bp, 90 bp, 75 bp) in column 10 and 11.

4. Discussion

The genus *Boophilus* consists of five species of which three were present in West Africa for many years: *R.* (*Boophilus*) *annulatus*, *R.* (*Boophilus*) *decoloratus* and *R.* (*Boophilus*) *geigyi*. In 2007, *R.* (*Boophilus*) *microplus*, an important vector of *Babesia bovis*, had been identified for the first time in this region where it now seems to cause major problems in dairy farms (Madder et al., 2007) (unpublished data). The introduction most likely occurred during one of the imports of Girolando cattle from Brazil, and this to improve local cattle breeds. The most important questions however, still remains unanswered: when was this tick introduced? It appears that private persons undertook several import actions the last decade.

Before 2007 and after the first import of Girolando probably in 2000, no published data are available of the presence or absence of *R.* (*Boophilus*) *microplus*. As outlined in the introduction, the morphological differences between some of the species of this genus are extremely small and some of the characteristics also seem to be variable. The difference between females of *R.* (*Boophilus*) *microplus* and *R.* (*Boophilus*) *annulatus* is almost exclusively based on the presence or size of the external spurs of the second and third coxae.

It was however observed that some specimens originating from Cameroon did show external spurs although the size and shape was somewhat different from those of *R.* (*Boophilus*) *microplus*. The hypostome dentition has been described to be variable as well, *R.* (*Boophilus*) *decoloratus* sometimes presents a dentition of 3.5×3.5 (Hoogstraal, 1956). In this study we also observed *R.* (*Boophilus*) *microplus* with a dentition of 4.5×4.5 (3 or 4 extra teeth in between the inner rows). The same applies to the shape of the spurs of the adanal plates of the males, which might be extremely variable in the case of *R.* (*Boophilus*) *microplus*. If the latter species would have been introduced in an area, it could have easily been overlooked, especially when ticks were damaged during collection or not cleaned thoroughly before identification. Especially female ticks are extremely difficult to identify and without males, which are easily been overlooked due to their small size, the presence of *R.* (*Boophilus*) *microplus* is extremely difficult to validate. In fact, morphological identification is far from specific, time consuming and requires sufficient expertise on different populations of the species in question. The molecular tool that was implemented represents a valuable aid for identification and would allow confirmation or disproval of previous or historical identifications. The first results of the PCR–RFLP tool yielded species-specific profiles and as all the screened populations of the same species originating from different countries showed consistent profiles, the test could therefore be used as a golden standard for tick identification of this genus in the region. The only restriction for the identification of *R.* (*Boophilus*) *microplus* might be the populations from Australia as recent studies have concluded the Australia strain not being able any more of producing fertile crosses with Africa and Latin American strains and being genetically different (Labruna et al., 2009).

The presence of *R.* (*Boophilus*) *microplus* in Ivory Coast was confirmed without any doubt. The samples that were collected in this country only presented *R.* (*Boophilus*) *microplus*, indicating that a recent introduction into this area seems unlikely or extremely successful. From all other countries in this study no *R.* (*Boophilus*) *microplus* were identified so far. It must however be mentioned that the number of samples screened was fairly low and consequently this should be confirmed. Future investigations are needed to determine the real extent of the presence of *R.* (*Boophilus*) *microplus* in Ivory Coast and neighboring countries and the effect on the transmission dynamics of *Babesia* spp. present in the area. To secure livestock production, improvement of cattle breeds and the import of exotic cattle breeds, the development of this reliable tool could offer opportunities for surveillance of ticks on cattle especially during importation.

B. Article 5: Foci report of indigenous *Dermacentor reticulatus* populations in Belgium and a preliminary study on associated babesiosis pathogens

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Key Words: *Dermacentor reticulatus*, *Babesia* spp., Tick, Belgium.

Ixodes ricinus (Linnaeus, 1758), the most abundant species in Northern Europe, is widely distributed and is mainly found in habitats with a high level of humidity (Hillyard, 1996). *Dermacentor reticulatus* (Fabricius, 1794) in northwestern Europe is more scarce and localized (Hillyard, 1996; Gray et al., 2009). The geographic range of this species extends from France and the southwest of the U.K. in the west through to central Asia in the east. Until recently, the French-Belgian border was considered to be the northern boundary of *D. reticulatus* in Western Europe (Heile et al., 2006). Recently, several reports have indicated that the geographic distribution of *D. reticulatus* is expanding. Indeed, over the last decade, *D. reticulatus* ticks have been collected from the environment in The Netherlands (Nijhof et al., 2007) and in different German states (Länder) (Dautel et al., 2006). Evidence of a change in the distribution of *D. reticulatus* is supported by the identification of canine babesiosis in regions of Germany (Heile et al., 2006; Barutzki et al., 2007), Hungary (Sreter et al., 2005), Switzerland (Porchet et al., 2007) and the Netherlands (Nijhof et al., 2007) not previously associated with this disease. In Belgium, adults *D. reticulatus* were found on a dog and on an unspecified animal species in the south of Luxembourg province, close to the French border, in 1945 and 1950, respectively (Fain, 1989). However, these historical observations did not provide any information on the origin of the tick or the travel history of the dog. More recently, low numbers of *D. reticulatus* were reported on dogs from one location in Belgium (Losson et al., 2003). In France, *D. reticulatus* is the most important vector of canine babesiosis (*Babesia canis*) (Martinod and Gilot, 1991) but it can also transmit other

protozoans, viruses and bacteria. The occurrence of autochthonous cases of canine (Losson et al., 1999) and equine (Mantran et al., 2004) babesiosis in Belgium during the last two decades suggests that this tick species may be present in this country.

Four locations in Belgium where *D. reticulatus* ticks were suspected to be present were identified: Beveren (referred to location 1) and Moen (location 2) in the northern Belgium, Mons (location 3) and Martilly (location 4) in southern Belgium (Figure 30).

The Beveren site (location 1, in the province of East Flanders) was identified in 2009 when *D. reticulatus* ticks were found repeatedly on a domestic dog that had no history of foreign travel. The site is a suburban marshland with a shallow artificial pond at its centre. It is situated between a railway track and a provincial road and is often used as recreational area for dog walking. The marshy area consists of a mixture of grasses while the dryer patches are occupied mainly by hornbeam (*Carpinus betulus*) and blackberry (*Rubus fruticosus).*

The site in Moen (location 2, in the province of West Flanders) was selected on the basis of photographs posted on an Internet site that collects fauna and flora observations (www.waarnemingen.be) showing what appeared to be questing *D. reticulatus* ticks that had been misidentified as *I. ricinus*. The site is a natural reserve of about 26 hectares and is located next to a canal that is accessible for recreational purposes. Location 3 is situated in Mons (in the province of Hainaut) and consists of fallow land flanked by a road. It is close to a leisure area that includes an artificial lake surrounded by a path that is extensively used for walking and exercising dogs. The vegetation consists of grasses, hawthorn, blackthorn (*Prunus spinosa*), brambles (*Rubus fruticosus*) and birch (*Betula pendula*). According to local veterinarians this area is a focus of canine babesiosis (Losson *et al*., 1999). Location 4 is situated in Martilly (in the province of Luxembourg). Two areas, referred to as "zone 1" and "zone 2", were monitored at this site. These zones are approximately 600 meters apart and are located in a rural environment consisting of woodland (mainly *Picea abies*) and pastures used for cattle grazing. In zone 1, which is situated close to a small stream, the trees were felled some years ago and the vegetation now comprises brambles, ferns (*Pteridium aquilinum*), blackthorn and jennets (*Genista scorpius*). Zone 2 comprises woodland consisting mainly of birch and oak (*Quercus robur*), with brambles and ferns also present. In both zones, roe deer (*Capreolus capreolus*) and red deer (*Cervus elaphus*) are present, as evidenced by the presence of numerous faecal deposits. Both zones were selected because *D. reticulatus* were found attached to two red deer hunted at these sites in February 2007.

Ticks were trapped by flagging with flannel cloth (Bram, 1978), in dry or sufficiently dry conditions. Location 1 was monitored on a weekly basis, from the end of February 2010 to the third week of November 2010. Location 2 was visited on only a few occasions. Locations 3 and 4 were sampled on a weekly basis during March-August 2010. Ticks were stored in 100 % ethanol immediately after trapping and morphologically identified using a standard key (Arthur, 1963). Tick DNA extraction was performed according to the proteinase K protocol (20mg/ml) of Boom et al. (1990). To discount potential false-negative results caused by polymerase chain reaction (PCR) inhibition and to validate the efficiency of the DNA extraction, an initial PCR test targeting the tick 16S rRNA gene was performed using the 16S+1 / 16S-2 primer set (Baumgarten et al., 1999). Only tick DNA-positive samples were further analysed for the presence of *Babesia* spp. The *Babesia* spp. genus-specific PCR was developed according to Casati et al. (Casati et al., 2006) and was designed to amplify a fragment of the 18S rRNA gene (Lempereur et al., 2011).

A total of 282 *D. reticulatus* adult ticks (98 males and 184 females) were collected from the 4 sites. The majority of the *D. reticulatus* ticks were found from early March and June with a peak in abundance in March although a few specimens were also found in July, August and October. Although *I. ricinus* is the most prevalent tick species in Belgium (Fain, 1990), only *D. reticulatus* was obtained in location 1 (Beveren), where a total of 139 (47 males and 92 females) adult ticks were collected. Weekly numbers of ticks collected indicated that questing activity was highest between March and April (Figure 31). Four of the 234 available tick DNA extracts remained negative for the tick 16S rRNA gene PCR test, despite the template DNA being diluted to 1:10 and 1:100, and consequently these samples were removed from the analysis. The remaining 230 DNA extracts were all found to be negative for *Babesia* spp. using the *Babesia*-specific PCR assay.

Although *D. reticulatus* is known to occur in Europe, it is considered to be scarcely distributed in the northwest of the continent (Hillyard, 1996) and has not been accurately recorded there. Recently, questing populations of this tick species were discovered by flagging in the Netherlands (Nijhof et al., 2007). *Dermacentor reticulatus* has also been documented in Germany (Dautel et al., 2006), Poland (Zygner et al., 2009), Austria (Sixl et al., 2003) and western Switzerland (Porchet et al., 2007). In this survey, indigenous questing populations of *D. reticulatus* were found in Belgium, supporting the findings of a previous survey indicating the presence of low numbers of feeding adults of *D. reticulatus* on dogs from one location in the country (Losson et al., 1999). As Belgium is situated between France

and the Netherlands, this study and others indicate that the species has extended its distribution area in recent years, moving from northern France to the Netherlands and populating Belgium in the process. Various factors such as landscape use, climate change, altered human activity and increases in host population density (such as in cervids) may explain this dissemination as Dautel et al. (2006) and Lindgren & Gustafson (2001) have summarized. Movements of people with animals carrying ticks and tick-borne diseases across borders may also explain this phenomenon.

Given its vectorial capacity, the spread of *D. reticulatus* may result in the occurrence of certain diseases in areas in which they have not previously been recorded. A similar observation was made in the Netherlands, where autochthonous canine babesiosis foci are known to exist (Matjila et al., 2005). No evidence of *Babesia* spp. was found in any of the tick samples analysed. However, the possibility that *D. reticulatus* can act as a vector for *B. canis* cannot be ruled out completely given the low prevalence of tick infection expected (Duh et al., 2004; Rar et al., 2005) and the relatively low number of ticks analysed in our study. In conclusion, the presence of indigenous populations of *D. reticulatus* was demonstrated for the first time in several distinct areas of Belgium. A more detailed widespread monitoring of *D. reticulatus* populations and screening for *Babesia canis* and other viral, rickettsial and bacterial pathogens are warranted in future studies.

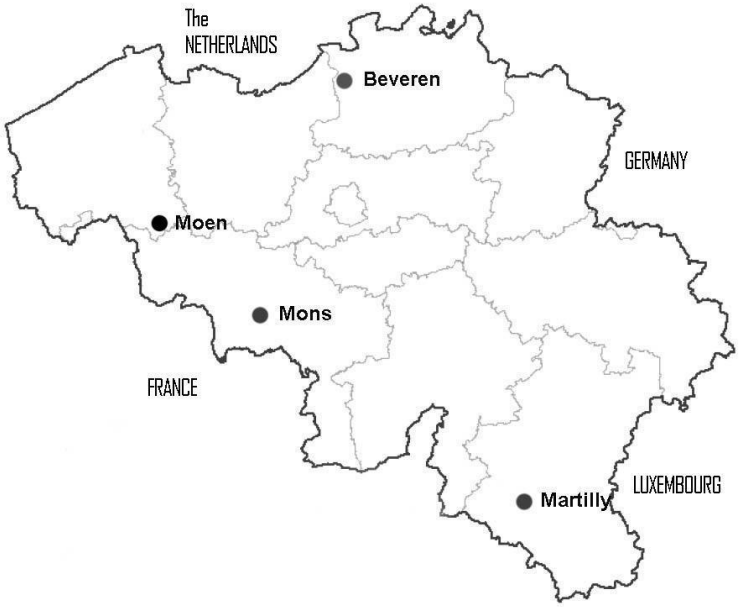

Figure 30: Map showing the four locations investigated in Belgium.

Beveren, location 1; Moen, location 2; Mons, location 3; Martilly, location 4.

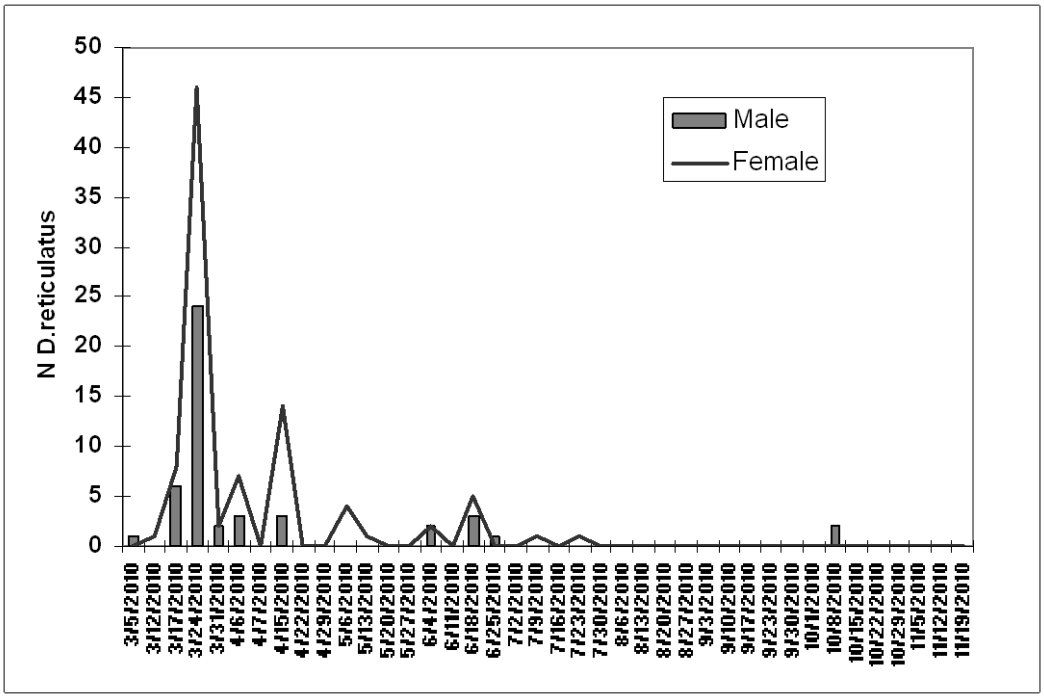

Figure 31: Numbers of *D. reticulatus* (male and female) flagged at location 1 (total n = 139).

V. Part III

A. Article 6: A retrospective serological survey on human babesiosis in Belgium

L. Lempereur, B. Shiels, P. Heyman, C. Saegerman, B. Losson and L. Malandrin.

Key Words: *Babesia* spp., Human, Zoonosis, Serology, Belgium.

1. Introduction

Babesiosis is a tick-borne disease caused by different species of intraerythrocytic protozoa classified within the genus *Babesia*. Interest in *Babesia* species with the potential to cause zoonotic disease has increased recently. Indeed, since the first recorded case in 1957 (Skrabalo and Deanovic, 1957), cases of babesiosis identified in humans have increased steadily (Hunfeld et al., 2008). In humans, clinical symptoms that develop early or during a mild *Babesia* sp. infection can be confused with malaria, showing high fever, headaches and myalgia. In acute clinical infection, anaemia, icterus and haemoglobinuria are characteristic and this condition can be fatal. In Europe, three species with zoonotic capabilities have been described. Most clinical cases have been attributed to *Babesia divergens*, a potentially zoonotic species commonly found in cattle, and have been documented primarily in splenectomised or immunocompromised individuals (Zintl et al., 2003), although some cases have recently been reported in young immunocompetent patients (Martinot et al., 2011). New species of *Babesia* have recently been described: *Babesia* sp. EU1 (proposed nomenclature *B. venatorum*) was identified for the first time in two asplenic human patients (Herwaldt et al., 2003), but has also been found to infect roe deer (*Capreolus capreolus*) (Duh et al., 2005; Bonnet et al., 2007). Characterisation of this species has essentially been based on comparison of the complete sequence of the 18S rRNA gene. *Babesia* sp. EU1 is placed phylogenetically in a sister group with *B. divergens* (Herwaldt et al., 2003). However, in addition to the

molecular differences, *Babesia* sp. EU1 is unable to reproduce in gerbils (*Meriones unguiculatus*) providing further evidence for recognition as a distinct species from *B. divergens* (Herwaldt et al., 2003). *Babesia* (*Theileria*) *microti* cases have been recorded in both spleen intact and asplenic patients, usually with a relatively mild clinical infection except in immuno-compromised or elderly individuals. *Babesia* (*Theileria*) *microti* is a natural parasite of microtine rodents and occurs mainly in the USA. However, a case of autochthonous *B.* (*T.*) *microti* infection has been confirmed in a patient afflicted with an acute myeloid leukaemia in Germany (Hildebrandt et al., 2007), and serological evidence of human *B.* (*T.*) *microti* infections in a number of different European countries has been reported (Hunfeld et al., 2002; Pancewicz et al., 2011). Clinical infection with *B. divergens* is considered as a medical emergency, with affected patients showing most of the characteristic signs, including a rapid, fulminating haemoglobinuria (Gray, 2006). Documented *Babesia* sp. EU1 infections showed similar but milder clinical manifestations compared to those due to disease caused by *B. divergens*.

In Belgium, *B. divergens* is known to be present in cattle in the south of the country (Famerée et al., 1977; Losson and Lefèvre, 1989; Lempereur et al., 2012a), but is considered to be absent from other parts of the country (Saegerman et al., 2007). Because of the recent first report of potentially zoonotic *Babesia* species in the *Ixodes ricinus* tick (Lempereur et al., 2011) together with an older report of a human clinical case in Belgium (Jadin and Giroud, 1981), a retrospective serological survey was performed. The aim of this study was to investigate the potential for zoonotic *Babesia* species to be in contact with humans known to have a history of tick bite and to evaluate the extent to which these contacts occur.

2. Materials and Methods

The human serum samples used in this study were collected and sent by physicians to the laboratory for vector-borne diseases in Neder-over-Heembeek, Belgium. In total, 200 sera from anonymous Belgian patients with history of tick bite and clinical symptoms compatible with a tick-borne disease were selected at random from samples obtained between 2005 and 2010. The retrospective serological survey focused on *Babesia* spp. was made according to the approval of the ethical committee of the Liège University Hospital (reference B707201010146).

An Indirect Fluorescent Antibody Test (IFAT) was used to screen for the presence of antibodies against *B. divergens, Babesia* sp. EU1 and *B.* (*T.*) *microti* in the 200 selected serum samples. A commercially available IFAT kit (Fuller Laboratories, USA) was employed to detect IgG to *B.* (*T.*) *microti*. According to manufacturer's specifications, samples are scored as positive at a 1:64 or greater dilution. Positive reactivity of test samples was achieved within a dilution range of 1:64 to 1:512. Positive and negative controls provided by the manufacturer and an additional positive control provided by the Reference Diagnostics Laboratory of the Center for Global Health (CDC, Atlanta, USA) were used to confirm accurate test performance.

The IFAT test for *B. divergens* was performed according to Chauvin et al. (1995), using a *B. divergens* Rouen87 clone F5, initially isolated from an acute human case of babesiosis, as the antigenic source. The *Babesia* sp. EU1 antigenic source was a clonal parasite line (C201A), initially isolated from roe deer and cultivated *in vitro* in sheep erythrocytes (Bonnet et al., 2007). Sera samples for both species were screened at dilutions of 1:8 and 1:16 with anti-parasite antibodies detected using fluorescein-conjugated goat anti-human immunoglobulin IgG (Sigma-Aldrich) at dilution of 1:50. IFAT results were scored by two independent readings, using Olympus BX60 microscope at x400. Images were captured using a Spot RT3 camera. Both bovine and human sera were used as positive and negative controls for the *B. divergens* IFAT. Positive control sera were collected from *B. divergens* infected cattle in France (Malandrin et al., 2004). The human positive control serum was a kind gift from Dr. Maija Lappalainen, Helsinki University (HusLab; Finland) and originated from a fatal clinical case attributed to *B. divergens* in a 53 year-old man (Haapasalo et al., 2010). This serum was first screened for reaction against *B. divergens* and *Babesia* sp. EU1 and the titre then evaluated against *B. divergens* antigen. Human negative control sera were selected from a previous study (Moreau et al., manuscript in preparation). Serum obtained from a sheep experimentally infected by *Babesia* sp. EU1 (clone C201A) was used as positive control for *Babesia* sp. EU1 test (Moreau et al., manuscript in preparation). Cross reactivity between IFATs for *B. divergens, Babesia* sp. EU1 and *B.* (*T.*) *microti* was tested by reaction of positive serum controls against the antigen preparation representing each species. The blue-fluorescent DAPI nucleic acid stain was applied to *B. divergens* and *Babesia* sp. EU1 positive IFAT slides to highlight the presence of parasite nuclei. Slides were mounted in 10µl of mounting medium containing 50% glycerol in PBS, 4, 6-diamindino-2-phenylindole (DAPI) at 1µg/ml and phenylenediamine at 1mg/ml.

3. Results

To validate the ability of IFAT to detect antibodies in serum derived from a human host infected with *B. divergens*, serum from a clinical case in Finland was used as positive control. The IFAT and serum titration demonstrated positive seroreactivity to *B. divergens* antigen with a titre of 1:512. Reactivity as indicated by the pattern of fluorescence was concentrated at the apical poles on the divergent angle of dividing parasites (Figure 32). The same localized pattern was observed with most *B. divergens* positive sera, with a difference in the fluorescence intensity (Figure 33). This pattern of reactivity was distinct from that obtained with positive control serum acquired from a bovine, where the pattern of reactivity showed an even distribution across the whole organism, with concentration of staining at the periphery in the absence of a detectable apical structure (Figure 34). DAPI staining showed that this structure did not colocalise with the nucleus of the parasite and it was concluded that the serum was reacting most strongly against antigen located to the apical complex (rhoptries/micronemes) (Figure 35).

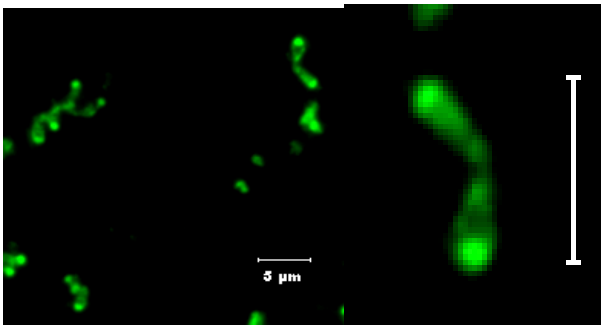

Figure 32: IFAT-reactivity of serum from a human clinical case of babesiosis in Finland (Haapasalo et al., 2010) at dilution 1:8 using *B. divergens* parasites as antigen. (scale = 5µm)

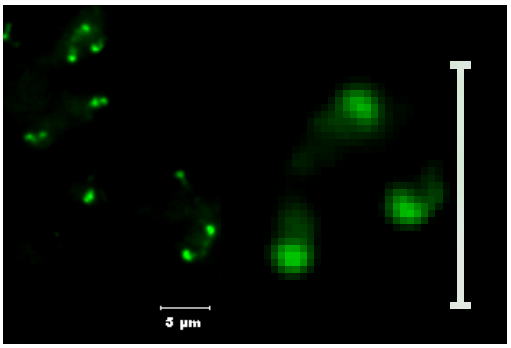

Figure 33: Reactivity of a positive serum from the Belgian panel of human tick bite associated samples at dilution 1:8 using a *B. divergens* IFA test.

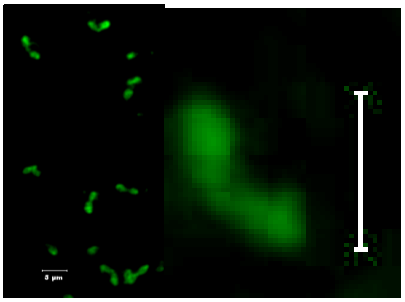

Figure 34: Reactivity of serum (dilution 1: 80) obtained from cattle in France infected with *B. divergens* (Malandrin et al., 2004) using a *B. divergen*s IFA test. (scale = 5µm)

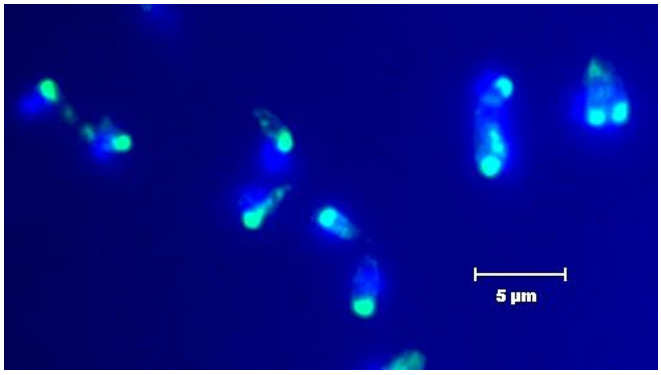

Figure 35: DAPI staining of serum from a human clinical case of babesiosis in Finland (Haapasalo et al., 2010) at dilution 1:8 using *B. divergens* parasites as antigen.

Positive reactivity against *Babesia* sp. EU1 antigen generated by positive sera from the samples of tick bite associated patients showed the same punctate pattern of fluorescence as obtained against *B. divergens* (Figure 36).

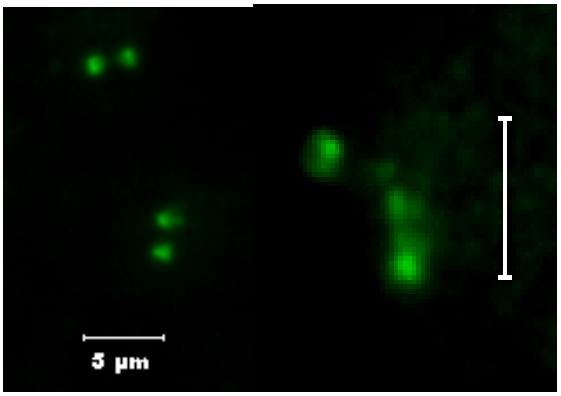

Figure 36: Reactivity of a positive serum from the panel of Belgian tick bite associated patient samples at dilution 1:8 using a *Babesia* sp. EU1 IFA test. Reactivity against the antigen was concentrated to a punctate structure at the apical pole on the divergent angle between two parasites. (scale = 5µm)

The pattern of reactivity to *B.* (*T.*) *microti* antigens in human showed an even distribution across the whole organism, with concentration of staining at the periphery in the absence of a detectable punctate structure (Figure 37).

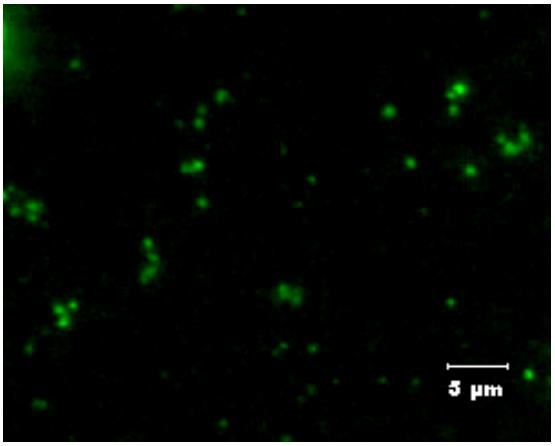

Figure 37: Reactivity of a positive serum from the panel of Belgian tick bite associated patient samples at dilution 1:64 using a *B.* (*T.*) *microti* IFA test.

To test for species specificity of the IFAT each positive control sera was cross-tested with each of the three antigen preparations representing the different species. As shown in Table 9, the results showed that at the titre designated for positive reactivity, each of the control serum reacted with antigen representing the *Babesia* species against which the serum was raised and

failed to react with either of the other two species. This result indicates that if a serum sample from the obtained panel reacted with more than one species by IFAT then it could be considered that the patient had been infected by two or more *Babesia* parasite species, at the same time or successively. Titration of *B.* (*T.*) *microti* positive samples ranged between 1:64 and 1:512. The low amount of human sera available for this study did not allow titration against *B. divergens* and *Babesia* sp. EU1 antigen.

The serological screening of the panel of 200 selected human sera from tick bite associated patients detected positive reactivity in 9% (18), 38.5% (77) and 42% (84) of the samples against *B.* (*T.*) *microti*, *B. divergens* or *Babesia* sp. EU1, respectively. The numbers of sera displaying positive reactivity were reduced if the cut-off for a positive reaction was taken at the 1:16 dilution (33% (66) and 40% (80) for *B. divergens* and *Babesia* sp. EU1, respectively). Four sera were scored positive for reactivity against *B.* (*T.*) *microti* and *B. divergens*, and two were found to react against *B.* (*T.*) *microti* and *Babesia* sp. EU1. Thirty-eight samples were positive for both *B. divergens* and *Babesia* sp. EU1. Two serum samples were IFAT positive for all three *Babesia* species (Figure 38).

	B. **(*T.*) *microti* Antigens (commercial kit)**	***B. divergens* Antigens (Rouen87 clone F5)**	***Babesia* sp. EU1 Antigens (Clone C201A)**
B. (*T.*) *microti* positive control from CDC (dilution	+	-	-
B. (*T.*) *microti* positive control from kit (dilution	+	-	-
B. divergens positive control from cattle (France) (dilution 1:80)	-	+	-
B. divergens positive control from human (Finland) (dilution 1:8)	-	+	-
Babesia sp. EU1 posive control from sheep (France) (dilution 1:32)	-	-	+

Table 9: Test for species specificity of the IFAT. Each positive control sera was cross-tested with each of the three antigen preparations representing the different species.

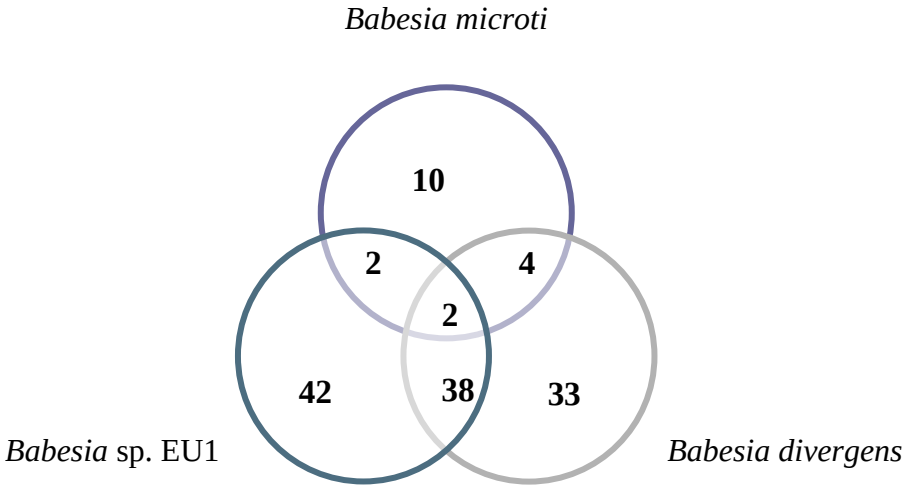

Figure 38: Results of the serological screening of the panel of 200 selected human sera from tick bite associated Belgian patients against *B.* (*T.*) *microti* (1:64 dilution), *B. divergens* and *Babesia* sp. EU1 (1:16 dilution).

4. Discussion

The retrospective serological survey performed in this study is the first in Europe to focus on 3 potential zoonotic *Babesia* species using a species-specific IFAT. The seroprevalence shown for *B.* (*T.*) *microti* (9%) is in agreement with other surveys in Europe using samples from patients suspected to be afflicted by another tick-borne disease (TBD) or with history of tick bites: 11.1% in Germany (Hunfeld et al., 2002) and 7.8% in Belgium (Jadin and Giroud, 1981). These results can also be compared to the 1.7% seroprevalence recorded for healthy blood donors in Germany (Hunfeld et al., 2002). In the Northeast United States where clinical babesiosis due to *B.* (*T.*) *microti* is considered to be an emerging disease, seroprevalence has been reported to range from 0.3% to 9.5% in Lyme disease patients (Leiby, 2011). The seroprevalence of 33% against *B. divergens* in the samples used in the current study is higher than a previous survey performed on samples from foresters in Slovenia (17.7%) (Rojko et al., 2008), while a recent survey in France generated a very similar level of positive samples

(Moreau et al., manuscript in preparation). While over estimation of the seroprevalence may be possible based on subjective scoring of positive Vs equivocal IFAT reactivity, steps were taken to minimize this by performing two independent readings and discarding results obtained at the lowest acceptable dilution. Furthermore, seroprevalence for tick-borne infections will clearly be skewed using samples generated from patients with a history of a tick bite compared to surveys incorporating samples form a wider range of human subjects. It has been suggested previously that in Europe human babesiosis may occur more often than previously indicated from the results of the few serological surveys that have been performed to date (Foppa et al., 2002; Hunfeld et al., 2002; Duh et al., 2007). Unfortunately, information about work, activity of the patients and geographical location within Belgium were not available. The obligatory anonymity of the patient and data confidentiality did not allow determination of risk factors that may be associated with tick contact and transmission of infection.

It is also the first time together with the survey done in France (Moreau et al., manuscript in preparation) that *Babesia* sp. EU1 seroprevalence has been evaluated using the proper *Babesia* sp. EU1 antigens.

No clear evidence of cross reactivity was observed across the antigen preparations employed for each IFAT when using positive control serum samples specific for each of the 3 species. Given the close phylogenetic relationship between *B. divergens* and *Babesia* sp. EU1, this result could be viewed as surprising. Duh and collaborators (2007) described an absence of cross reactivity of serum against *B. divergens* with *B.* (*T.*) *microti*, while cross reactivity of sera between *B. divergens* and *Babesia* sp. EU1 was reported (Herwaldt et al., 2003; Duh et al., 2007). It may be that IFAT positive results have scored potential *B. divergens* and *Babesia* sp. EU1 co-infection as cross recognition. In the present study, a clonal line of *Babesia* sp. EU1 cultivated *in vitro* in sheep erythrocytes has been used as the antigenic source and 46 patients positive for *Babesia* sp. EU1 were scored as negative for *B. divergens*. The results may indicate that the antigenic epitopes of these parasites predominantly recognized by sera from the selected panel have diverged significantly. A lack of cross reactivity would lend further support for the description of *Babesia* sp. EU1 as a new species of *Babesia*.

The number (38) of potential co-infections identified in this study by co-recognition of both *B. divergens* and *Babesia* sp. EU1 was high. Frequent co-infections with *B. divergens* and *Babesia* sp. EU1 may be possible, as these species share the same tick vector and co-

transmission of infection may occur. Indeed, concomitant or serial infection of pathogens seems to be common in tick exposed patients (Hunfeld et al., 1998). Nevertheless, further studies are required to show that results based on recognition of both species by an antibody test, correlate with molecular data validating co-infection of the mammalian host.

Differences in IFAT reactivity pattern associated with detection of *B. divergens* have been reported in different studies (Hunfeld and Brade, 2004; Duh et al., 2007) and although no difference was seen when antigen from human and bovine origin were compared, a higher end-point titre was obtained using antigen of human origin (Duh et al., 2007). With human sera reacting against *B. divergens* parasites, reactivity was found to be predominantly against the apical complex, while this was not the case using bovine serum on the same antigen preparation. This IFA pattern may suggest the presence of immunodominant antigens localized in the apical complex of the parasite. However, the strong staining at the periphery of the parasite obtained with bovine serum could obscure a weaker pattern of reactivity at the apical poles. *Babesia divergens* merozoite surface antigen (Bd37) is known to be a major immunodominant protein, but why this immunodominant antigen could be detected by immunoblotting with bovine serum samples but apparently not human (data not shown). However, polymorphism of Bd37 has been reported (Carcy et al., 2006). Therefore human antibodies could react with divergent Bd37-related epitopes that were not represented by the bovine *B. divergens* genotype(s) used for antigen generation, and then fail to detect either the parasite periphery by IFAT or a dominant polypeptide of 37 kDa by immunoblot. Ideally further experiments should be performed comparing the reactivity pattern of the sera used in the current study against *B. divergens* produced in human as well as of bovine origin, but the scarcity of clinical human cases makes this difficult.

At present IFAT, although not ideal, is the best available test to evaluate the occurrence of *Babesia* spp. transmission to human, since these parasites are usually eliminated rapidly by human immune system leaving only serological trace. Implementations of other serological tests such as ELISA or Western Blot were attempted in this study but the major immunodominant merozoite surface antigen failed to be recognized (unpublished observation). To fully evaluate the level of exposure of the human population with *Babesia* parasites in Belgium the development of a "home-made" IFAT test based on a European strain of *B.* (*T.*) *microti* is required. The paucity of European *B.* (*T.*) *microti* human clinical cases, however, means that routine provision of a standard antigen would be extremely difficult without an available culture system. Moreover, the use of the commercial IFAT kit

based on *B.* (*T.*) *microti* American strain imposes significant costs and precludes the use of this test for large scale serological survey, which may lead to an underestimation of *B.* (*T.*) *microti* infection. Such an underestimation could have potential consequences given that it is now recognized that contamination of human blood with *B.* (*T.*) *microti* is a potential risk during blood transfusion and *B.* (*T.*) *microti* infections are now notifiable in the USA (Leiby, 2011).

In summary, the presence of the three potentially zoonotic species of *Babesia* has been confirmed together with evidence for infection of humans, reported for the first time in Belgium, with *B. divergens*, *Babesia* sp. EU1 and *B.* (*T.*) *microti*. Thus in this country as in other European countries babesiosis has to be considered as a threat for human health especially in splenectomised and immunocompromised individuals. Preventive actions, such as improved awareness of physicians and development of better diagnostic tools should be helpful to prevent clinical cases and assess the true risk to public health.

VI. General Discussion

The aim of the research performed in this thesis was to generate information on the general situation of babesiosis in Belgium, with emphasis on investigation of potential zoonotic species of the *Babesia* parasite. Evaluation of *Babesia* present in tick generated evidence for species previously unreported in Belgium; *Babesia* sp. EU1 and *Babesia capreoli*. *Babesia* sp. EU1 was proposed by Herwaldt et al. (2003) to be a new species, based on a 31 base pairs difference in the sequence of the 18S rRNA gene and the inability of *Babesia* sp. EU1 to infect and kill gerbils (*Meriones unguiculatus*) after experimental infection. Nevertheless, based on morphological criteria, vector identity (Becker et al., 2009; Bonnet et al., 2009), and host range, *B. divergens* and *Babesia* sp. EU1 could be divergent members of the same species with the potential to undergo sexual recombination. *Babesia* sp. EU1 has been recorded several times in different surveys, with evidence of molecular differences relative to *B. divergens* (Table 8), and the phylogenetic tree (Figure 25) predict that *B. divergens* and *Babesia* sp. EU1 belong to the same cluster group but can be considered to be different species. Absence of serological cross reaction between *Babesia* sp. EU1 and *B. divergens* using species specific control sera and antigen preparations (article 6) also indicates that antigenically significant divergence has occurred, as might be expected following a species split, and could be taken as supporting evidence for definition of *Babesia* sp. EU1 as a new species. However, given the known ability of parasites to generate significant antigenic diversity and variation within a species (Chauvin et al., 2009), it would be unwise to use this data as conclusive evidence.

Discrimination between *B. capreoli* and *B. divergens* is also difficult morphologically, serologically and molecularly. Moreover, they share the same tick vector, *I. ricinus*, and present an overlapping vertebrate host range. These species do, however, differ in their host specificity. A 3 base pairs difference between species was identified over the entire 18S rRNA gene sequence and this is often used to discriminate these species. Internal transcribed spacers 1 and 2 (ITS 1, ITS 2) genes have also been used for discrimination and revealed high level of similarity between species (Schmid et al., 2008). Except from 18S rRNA, few other genes have been sequenced in *B. divergens* and they are usually highly conserved (β tubulin, hsp, subtilisin) (Caccio et al., 2000; Montero et al., 2006; Montero et al.,

2008). Merozoite surface proteins such as Bd37 have been described in *B. divergens* and such proteins are known to be encoded by genetically variable genes due to host immune selection pressure (Carcy et al., 2006). The othologue of Bd37 was described in *B. capreoli* (Bcp 37/41) and PCR-RFLP applied to this gene may prove useful to molecularly discriminate both species. Nevertheless, intra-specific variability of merozoite surface antigen genes must be assessed before a species-specific PCR based on this gene can be developed and deployed (Sun et al., 2011).

Babesia sp. EU1 and *B. capreoli* are known to have cervids as mammalian host (Duh et al., 2005; Zintl et al., 2011), but the role of deer as a reservoir for *B. divergens* is still controversial. *Babesia divergens* has been found in roe and red deer (Duh et al., 2005; Garcia-Sanmartin et al., 2007; Zintl et al., 2011). However, since it is difficult to distinguish *B. divergens* from *B. capreoli,* morphological misidentification is possible, and further molecular based studies with a high level of species-discrimination are required.

As deer can probably be a reservoir host for *B. divergens,* it may be that cattle play the same role for *Babesia* sp. EU1 in natural habitats characterized by deciduous forests with large populations of wild ruminants. This hypothesis has yet to be proven: indeed experimental validation of cattle acting as a reservoir for a *Babesia* species of deer has only been tested for *B. capreoli* (Adam et al., 1976; Gray et al., 1990; Malandrin et al., 2010) If it can be demonstrated that cattle act as a reservoir for *Babesia* sp. EU1 it would raise implications for the epidemiology and control of this zoonotic parasite.

Following discovery and confirmation of the presence of potential zoonotic species, evaluation of infection rate in ticks collected from their respective vertebrate hosts was performed (study 1, 2 and 3). Unfortunately, collection of ticks from rodents was not possible due to time and budget limitations. Tick collection was also achieved from specific environments and seroprevalence of babesiosis was evaluated for bovines and humans. The results are summarized in Table 10 and the projected geographical distribution of *Babesia* spp. found in Belgium during the different surveys is shown on Figure 39.

Tick infection rate from different hosts	***Babesia* sp.**	***B. capreoli***	***B. divergens* /*B. divergens*-like**	***B.* (*T.*) *microti***	***Babesia* sp. EU1/*Babesia* sp. EU1- like**	***Babesia* spp.**
Cat/Dog	0.24% (2)	/	/	0.36% (3)	0.71% (6)	1.31% (11)
Deer	0.39% (4)	0.2% (2)	0.78% (3/5)	/	1.37% (11/3)	2.7% (28)
Bovine	/	/	/	/	14.6% (23)	14.6% (23)
Environments	/	/	/	/	7.9% (34)	7.9% (34)
Seroprevalence						
Human			33%	9%	40%	59% (118)
Bovine			14.3%			14.3%

Table 10: *Babesia* infection rates in ticks collected from different vertebrate hosts and environments. Seroprevalence of babesiosis evaluated in bovines and humans. Number of positive samples is given in brackets.

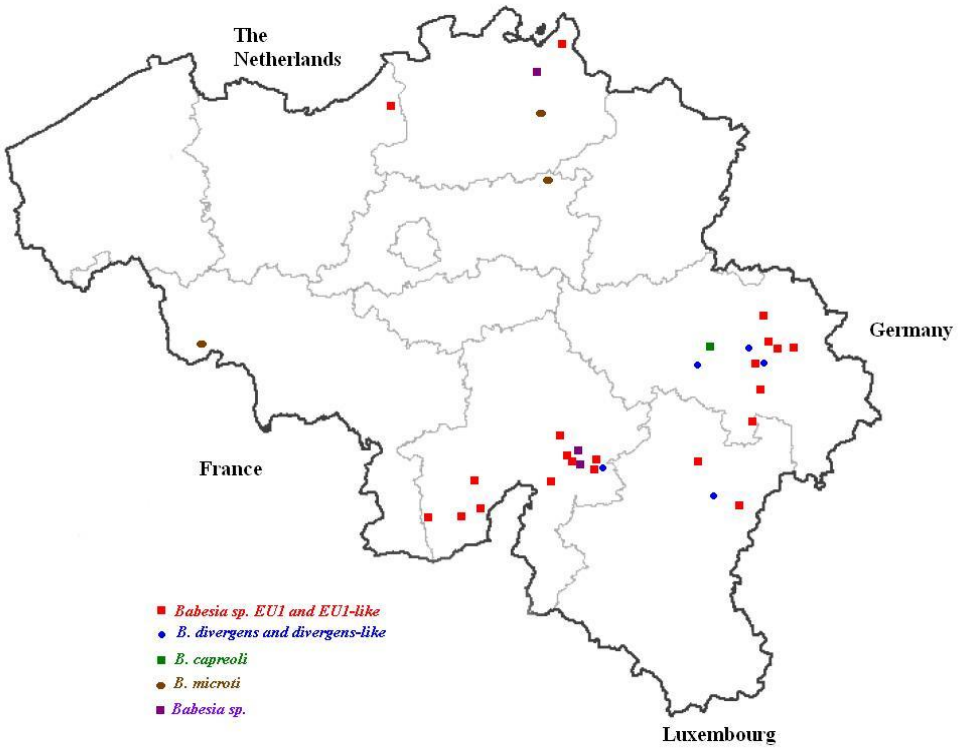

Figure 39: Distribution of *Babesia* spp. positive samples in Belgium. Results from the different studies were combined.

Tick infection rates, in general, are mostly in agreement with other surveys, although some marked differences were found compared to other studies based on the same or different hosts

or environments. These disparities could be due to a multiplicity of factors such as collection technique, environmental factors influencing ticks density, the stage and biology of collected ticks, host density, and targeted population sampling. It should be borne in mind before making a strict comparison that all of these factors could influence the results of different studies. Illustration of this can be made using the example of the bovine targeted population described in article 2 since the result showed a higher infection rate and seroprevalence than expected, probably due to farm locations in known endemic areas, high density of cervid populations in the study area and to the different tick stages collected.

Investigation of geographical expansion of a tick vector has been approached in this thesis by study of *D. reticulatus* in Belgium and *R.* (*Boophilus*) *microplus* in West Africa. Despite the failure to record *Babesia* infection in tick populations showing expansion in both Belgium and Ivory Coast, both situations highlight the potential risk of tick-borne diseases emerging in a previous uninfected area. It is generally accepted that the risk of tick-borne diseases is increasing and has potential to become of greater significance due to climate change, landscape use, increases in host population density, and animal movements such as livestock importation or people moving animals carrying ticks and tick-borne diseases across borders. Additional factors, such as altered human recreational activity and an increasing number of immunocompromised patients may also lead to a greater level of tick-borne disease.

Expansion of reservoir host populations could also increase, directly or indirectly, the risk of babesiosis. Such hosts promote maintenance of the parasite, allow transmission to the next generation of feeding ticks and advance geographical vector extension. Since 1975, wild ruminant populations have doubled in Southern Belgium, despite an increase in recreational hunting activity by humans. A preference for shooting males as trophies, artificial feeding of animals in winter and low level of hunting roe deer are considered to be probably the most important causes (Libois, 2006; SPW - DGO3 Agriculture-Ressources naturelles et Environnement, 2011). Such a situation has the potential to alter the balance between the parasite and its potential hosts, particularly humans where infection is most likely to originate from an animal reservoir.

Ixodes ricinus is also the vector of other human and animal pathogens such as *Borrelia burgdorferi sensu lato* and *Anaplasma phagocytophilum*. Co-infections with *Babesia* spp. are probably fairly common as demonstrated for *Borrelia* spp. (see article 1) or *Anaplasma phagocytophilum* (see article 2) and could enhance the severity of the protozoal infection in

humans and animals (Welc-Faleciak et al., 2010; Hildebrandt et al., 2011b). The high number of co-infection with *B. divergens* and *Babesia* sp. EU1 in human, predicted by serology (see article 6), implicate possible common transmission by a tick infected with both species. Indeed, concomitant or serial infection with tick-borne pathogens seems to be common in tick exposed patients (Hunfeld et al., 1998). The fact that *Babesia* sp. EU1 was the only species found in both feeding and questing *I. ricinus* in a region previously identified as endemic for *B. divergens* (article 2) suggest the possibility of co-infection by both species. Although the study suggests that at the time of sampling *Babesia* sp. EU1 infections would predominate. Further work investigating potential reservoir hosts and the epidemiology of both *Babesia* species in this region are required. It is known that co-infection of livestock, synchronous or not, with tick-borne diseases can exacerbate disease morbidity and reduce host fitness (Zintl et al., 2003). Furthermore recent studies have provided evidence that interaction between tick-borne pathogen populations can influence tick-borne disease epidemiology. This can be manifest in a positive or negative manner and alter pathogen prevalence, pathogen transmission and host susceptibility (Ginsberg, 2008; Telfer et al., 2010). Therefore, to gain fuller insight into the factors that influence risk from tick-borne disease, future studies should attempt to capture data on interacting parasite communities rather than individual parasite species.

Zoonotic babesiosis is primarily caused by 3 different *Babesia* spp., and for the first time evidence for infection of humans in Belgium with *B.* (*T.*) *microti, B. divergens* and *Babesia* sp. EU1 has been reported in this thesis (see article 6). Epidemiology or pathogenicity of *B.* (*T.*) *microti* in humans seems to be different between the USA and Europe, with more clinical cases reported in the USA. A number of hypotheses have been put forward to explain this difference such as circulation of different *B.* (*T.*) *microti* strains (that possess different levels of virulence) as suggested by molecular phylogeny (Figure 25), or the fact that different tick species act as vectors in the USA and Europe. Nevertheless, seroprevalence in the Northeast USA has been reported to range from 0.3% to 9.5% in Lyme disease patients (Leiby, 2011) and could be compared with the result of our study (9%, article 6) or the study of Hunfeld et al. (2002) (11.1%) in patients with a history of tick bites in Germany. In Europe, the real prevalence of *Babesia* infection in humans is most likely to be underestimated because of subclinical infections (Persing et al., 1992) or masked by co-infection with other tick-borne pathogens such as *Borrelia* spp. but poor availability of diagnostic tests and a lack of physician awareness could also be factors. Such a situation may have contributed to a failure

to previously diagnose clinical babesiosis cases in the USA. The disease is now recognised as an emerging condition and improved diagnostic tests for blood donor screening and increased awareness of clinicians are thought to be necessary.

Suitable diagnostic tools are of great importance for both *Babesia* and tick species discrimination. *Babesia* spp. identification is not particularly easy; the fact that it can be based on slight differences in the 18S rRNA gene sequence encourages recommendation that the whole 18S rRNA gene is sequenced. Implementations of new molecular tools that provide more rapid speciation are required. Moreover further study of genomic polymorphism, and possibly transcriptomes linked to virulence traits, may also be necessary if some strains within a species turn out to be more associated with clinical disease than others. Although PCR is a very sensitive method, several compounds in blood such as haemoglobin and lactoferrin have been suggested to be PCR inhibitors. This proof that in a *Babesia* negative sample from ticks, dilution and confirmation of amplification of the tick 16S rRNA gene was generated in our study (article 1). High proportion of erythrocytes are present in engorged female ticks and removal of the bloodmeal has been suggested as an approach to mitigate PCR-inhibitory effects (Dharmarajan and Rhodes, 2011). Whether such inhibitors would compromise a pooling system of samples requires testing because this would generate both time and cost benefits, but may reduce or significantly compromise sensitivity.

Cross reactivity is the main constraint for serological diagnosis of *Babesia* spp. together with a lack of human derived parasites for generation of antigen preparations, which hinders assay development and implementation of other serological test for results confirmation. Work is also needed to investigate further the antigenic relatedness of *B. divergens* and *Babesia* sp. EU1 and also the relative repertoire of antibodies generated in humans and reservoir hosts against the same parasite species e.g. *B. divergens* (see article 6). Major immunodominant antigen candidates should be evaluated as they may improve diagnostic test specificity, as long as they show a degree of conservation within a species. At present development of serological diagnoses are likely to be essential in order to study and prevent babesiosis, especially potential transfusion-transmitted *Babesia* spp.

VII. Conclusions and Recommendations

Babesiosis has to be considered as a relative threat for the health and welfare of livestock, companion animals and humans, especially in elderly, co-infected, splenectomised or immunocompromised patients.

Tick control should be implemented, especially on exported livestock and animals moving across borders. These factors have probably promoted the geographical expansion of *D. reticulatus* in Belgium and *R.* (*Boophilus*) *microplus* in West Africa and increase the potential risk of tick-borne disease.

Integrated pest management including different measures to control tick is to be required. Daily checkup and tick removal is recommended for companion animals. Acaricide treatment scheme should also be implemented for companion animals and for livestock especially in naïve adult cattle or in non *Babesia* spp. endemic areas. This anti-tick treatment has to be moderate on calves in order to promote contact with *Babesia* spp. infected ticks during innate resistance period (3-9 months old). Research on development of more ecologically sustainable solutions for tick control should be promoted. For humans, preventive action should be implemented to minimize the risk of contracting tick-borne disease. Use of repellent and body examination could be recommended respectively before and after walking activity in tick habitats especially for at risk people.

Nevertheless, one of the most useful strategies remains dissemination of relevant risk information to the medical community and the general public. A more detailed widespread monitoring of vector populations, particularly in at risk disease free region/countries, should be made and screening for *Babesia* spp. and other viral, rickettsial and bacterial pathogens are warranted in future studies in order to find the most preventive strategy adapted for each epidemiological situation. Epidemiological surveys should be standardised to allow strict comparison of the results. Large scale studies should be extended to all potential hosts and their associated risk factors involving collaboration between medical and veterinary researchers where necessary. This is relevant even to Belgium because expansion of reservoir host populations such as wild ruminant populations could also increase the risk of tick-borne disease. For such a scenario, hunting regulations may have to be adjusted in order to reduce

game populations if risk assessment shows that an actual increased threat from disease justifies this action. Studies on tick-borne diseases should attempt to analyse and capture data in a global approach involving hosts and vectors interacting in different environments in order to fully understand risk factors and infection dynamics. Moreover, companion animals who live in close contact to humans and are exposed together to *I. ricinus* ticks and the pathogens they carry could be considered as sentinels for tick-borne diseases such as borreliosis, anaplasmosis (Olson et al., 2000; Duncan et al., 2005), and babesiosis. Collaboration between medical and veterinary profession should be again encouraged following the concept "One World One Health".

General practitioners should be aware of the existence of this malaria-like disease and the sector of the population most at risk. Splenectomised and immunocompromised patients should be made aware of the risk they incur when they are walking in endemic tick infested habitats.

Rapidity and specificity of diagnosis is an essential step in order to improve vital pronostic of patient. General practitioners and veterinarians have to be able to make an appropriated diagnosis as required. Potential co-infections have to be taken into account especially when non responding specific treatment occurs. Improved standard diagnostic tools are clearly required and will help notably to control the transmission of human infections, as will give an understanding of at risk areas within countries where tick populations and reservoir hosts show uneven distribution. Further research on human babesiosis in Europe should be encouraged, particularly epidemiological serological survey on blood donors and "at risk" populations. Identification of risk factors of transmission are required and molecular based studies with a high level of species-specific fidelity can be used to determine with certainty the reservoir hosts that are infected with the *Babesia* species/genotypes responsible for human disease.

Treatment regime has to be promptly initiated and in livestock has to be adapted to each economical and epidemiological situation. In Europe, imidocarb diproprionate (Cabesia®) is the only allowed specific treatment against Babesiosis and relatively long withdrawal periods (cattle milk: 2 days, meat: 28 days; horse: 60 days) has to be respected. Its supplying can be difficult in some countries and its use as prophylactic treatment could be substituted by long - acting oxytetracyclin. This would have the advantage of covering other potential tick-borne diseases but the disadvantage of a longer milk withdrawal period (7 days) and a potential

induction of antibioresistance. Supportative treatments (perfusion, blood exchange…) should be encouraged and use of corticosteroids should be proscribed as course of infection is often speeded up by their immunosuppresion effect.

Evolution of the epidemiological situation of Babesiosis depends on numerous factors and seems to be difficult to predict. In humans, risk of an increasing clinical case number would be mostly linked to a rise of immunocompromised patients. Nevertheless, a change in parasite virulence and host specificity can never be excluded. In Belgium, bovine babesiosis encountered a drastic rise during the 70's (Famerée et al., 1977) and clinical cases were reported from all provinces of Wallonia. However, since then no clinical bovine Babesiosis was found from other part of the country, except two cases most probably imported (Bouquet, 1962; Everaert et al., 2007). Although bovine Babesiosis study from this thesis (study 2) recorded a slightly higher seroprevalence, this proposition needs to be confirmed by a larger scale survey in order to extrapolate to all Belgium.

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X. Appendix