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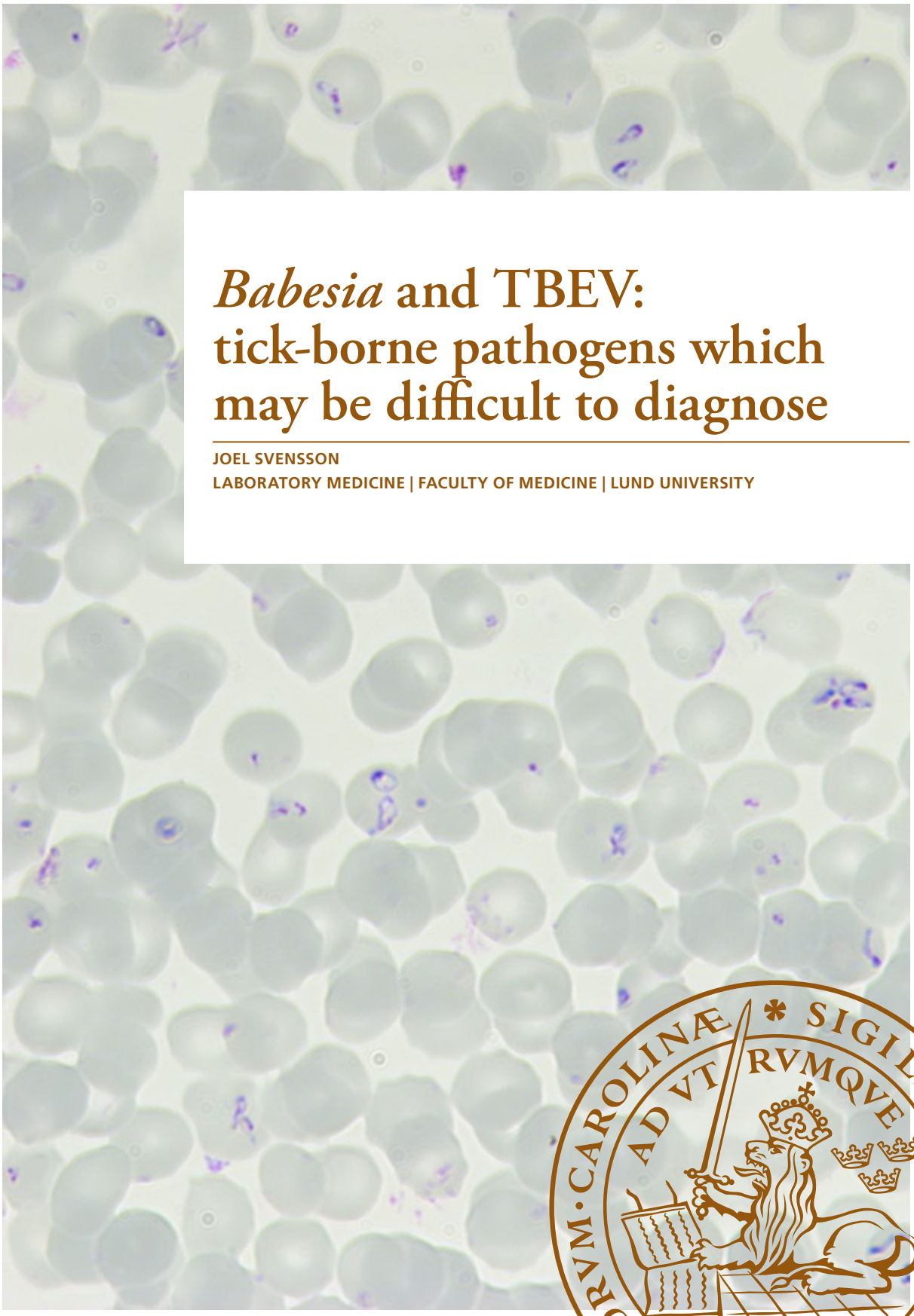

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LUND
UNIVERSITY

DOCTORAL DISSERTATION

Doctoral dissertation for the degree of Doctor of Philosophy (PhD) at the Faculty of Medicine at Lund University to be publicly defended on 15th of June at 13.00 in The Segerfalk Hall, Biomedical Centre (BMC), Sölvegatan 19, Lund.

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Abstract:
Babesia and Tick-borne Encephalitis Virus (TBEV) are tick-borne pathogens with an increasing interest in Sweden during the last decades. *Babesia* is an intraerythrocytic parasite with a similar appearance and life cycle as malaria (*Plasmodium*). More than 100 species of *Babesia* have been described, and while some of them are well known to cause disease among cattle in Sweden (redwater fever), some of them have also been seen to cause human babesiosis. In the US especially *Babesia microti* is a well-known pathogen and in Europe *Babesia divergens* have caused most known human cases. Human babesiosis have different clinical manifestations, from milder to more severe with potentially fatal cases. Fever and myalgia are common symptoms and risk factors are immunosuppression and previous splenectomy. Another risk of acquiring the disease is through blood transfusion.

Different methods are used to detect the *Babesia* parasites, such as direct techniques for example microscopy of blood smears and PCR; and indirect techniques such as antibody assays for example Enzyme-linked immunosorbent assay (ELISA) and Indirect Fluorescent Antibody Assay (IFA). For *Babesia divergens,* there has been a lack of easy-to use indirect assays.

In our first work we showed with an IFA technique that among a group of individuals with confirmed antibodies against *Borrelia burgdorferi*, 16% had antibodies against at least one of the *Babesia* spp. This was significantly more compared to a healthy control group (2.5%), and it showed that *Babesia* is more common in southern Sweden than has been assumed before.

We have also (study 3) used blood from an infected cow with *Babesia divergens,* transferred it to a cell culture where the parasites could proliferate and thereafter, they have been used in an ELISA that we created ourselves. This assay showed a higher seroprevalence among *Borrelia* infected humans and good consistency with IFA.

TBEV is a disease which is getting more attention in Sweden. With potentially severe symptoms and a life-threating course, it is also noticed to increase in southern Sweden. In our collected cohorts of tick-bitten individuals, we have seen high prevalence's of antibodies against TBEV (study 2). This motivates an increased awareness for the pathogen in the region and can also indicate that subclinical or milder infections can occur.

In conclusion, *Babesia* and TBEV should get more attention in Swedish healthcare as potentially severe and fatal pathogens. More laboratory diagnostic opportunities should be available. The risk of *Babesia* as a blood transfusion transmitted pathogen should also be considered.

Key words: Tick-borne diseases, *Babesia divergens, Babesia microti*, Tick-borne encephalitis virus, Enzyme-linked immunosorbent assay, Indirect fluorescent antibody assay, Human babesiosis, *Ixodes ricinus*, Sweden

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For Jenny, Alma, and Elias

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Sammanfattning på svenska

Babesia är en parasit som i Sverige hittills varit relativt okänd hos människa och som kan orsaka sjukdomen babesios. Parasiten kan framför allt spridas via fästingar och är en av de mindre välkända fästingburna smittorna i Sverige.

Babesia har länge varit allmänt bekant inom veterinärmedicinen och kan hos kor orsaka "sommarsjuka" eller ”blodhalning”, med symtom som röd urin, spontan abort och död. Den är speciellt välkänd inom vissa veterinärdistrikt med återkommande fall, där djur ofta behandlas direkt vid uppkomna symptom utan vidare diagnostik eller utredning.

Livscykeln för *Babesia* är snarlik den för malariaparasiten. Detta då båda parasiterna vid infektion invaderar och förökar sig inuti den röda blodkroppen, och när man tittar på dem i mikroskop är de mycket lika varandra. En stor skillnad är att det för *Babesia* är en fästing som överför sjukdomen och för malaria är det en mygga.

Symptomen hos människor kan vid en infektion sträcka sig från inga symptom alls, till döden. Episoder av feber som ofta liknar influensa med smärta i kroppen, huvudvärk och feber är förmodligen de vanligaste symtomen hos annars friska människor. Detta påminner om infektion av malaria. Hos personer med nedsatt immunförsvar eller hos de som saknar mjälte är sjukdomen mycket farligare och kan vara dödlig med njursvikt, blodbrist och allmän organsvikt. Behandlingen består av en kombination av antibiotika och malarialäkemedel såsom kinin. Det kan ofta krävas en lång behandlingstid för att bli av med parasiterna.

Efter att parasiter har förts in i blodbanan av fästingen, utvecklas de och växer till i de röda blodkropparna. Andra smittvägar för *Babesia* är via röda blodkroppar vid blodtransfusioner. Denna smittväg är välkänd i USA och där screenas blodet för *Babesia* i visa delstater. Det har hittills inte setts lika många fall av transfusionssmitta som för fästingöverförda infektioner, men det kan vara extra allvarligt när det väl sker. En orsak är att patienter som emottar en blodtransfusion ofta redan är sjuka och än mer mottagliga för en infektion. *Babesia* bedöms vara en av de potentiellt farligaste smittorna i USA vid transfusioner av röda blodkroppar.

Det har beskrivits över 100 olika arter av *Babesia*. I USA dominerar arterna *Babesia microti* och *Babesia duncani* och det rapporteras drygt 2000 fall per år vilket är ungefär en tiondel av antalet rapporterade fall av borreliainfektioner. Cirka hälften av patienterna behöver vårdas inneliggande på sjukhus. *Babesia* är koncentrerat till

vissa delstater på östkusten och i mellanvästern. I Europa har fall med sjukdom hos människa framför allt varit med arterna *Babesia divergens, Babesia microti* och *Babesia venatorum*. I Sverige har enbart ett fåtal patienter med babesios beskrivits.

Metoder för att direkt detektera parasiten är till exempel ljusmikroskopi och molekylärbiologiska metoder, och indirekta metoder som undersöker antikroppars förekomst har idag alla olika brister i form av känsligheten för att detektera smittan och också låg tillgänglighet inom rutinsjukvård.

Vi har i vår första studie (studie I) visat att 16% av personer som hade antikroppar mot *Borrelia* också hade antikroppar mot *Babesia*. Det vill säga bland många som hade utretts för en borreliainfektion, hade också många haft en exponering för *Babesia*. Detta jämfördes med en frisk kontrollgrupp, där drygt 2% hade antikroppar mot *Babesia*. Antikropparna var mot arterna *Babesia divergens* och *Babesia microti*. Det var en förvånansvärt hög andel med antikroppar mot *Babesia microti,* då denna art tidigare framför allt varit uppmärksammad i USA. Resultaten tyder på att *Babesia* kan vara vanligare än vad vi tidigare har trott och bör få ökad uppmärksamhet. I många fall kan man tänka sig att infektionen har ett självläkande förlopp men det finns också en överlappning av symtomen gentemot *Borrelia*. Det behövs också att fler riktade utredningar görs i aktuella patientfall där relevanta symptom och riskfaktorer föreligger.

I en annan studie (studie III) har vi med hjälp av blodprov från en smittad ko kunnat odla babesiaparasiterna i en cellodling. Med hjälp av proteinantigen från parasiterna har vi kunnat utveckla en laborativ analys för att detektera och känna igen antikroppar för *Babesia* (ELISA). Med hjälp av antikroppsmetoden har vi sett att hos tidigare fästingbitna personer (tidigare borreliainfekterade personer) så hade en större del av dessa jämfört med en kontrollgrupp antikroppar mot *Babesia divergens,* och när vi jämförde med en annan antikroppsbaserad metod (IFA) så var det god överensstämmelse, men ELISA är enklare att använda.

TBEV (tick-borne encephalitis virus) är ett fruktat virus som kan orsaka inflammation i hjärnvävnaden, så kallad encefalit. Infektioner med milda eller subkliniska förlopp har också beskrivits. Sjukdomen har under de senaste decennierna setts i ökad omfattning i Skåne, dock fortfarande på relativt låga nivåer. I ett projekt (studie II) såg vi att antikroppsnivåerna var förvånansvärt höga även i områden där infektionen inte setts tidigare. Vi såg också lägre nivåer av antikroppar bland personer mellan 50–69 år jämfört med grupper med yngre respektive äldre personer. Detta kan tyda på högre nivåer av smitta hos yngre vuxna och äldre, vilka kan ha olika risker för att utsättas för smitta i form av vanor att vistas ute i naturen. Förmågan att bilda och bevara antikroppar kan också spela in och förändras ofta med det sämre med åldern.

Sammanfattningsvis har vi visat att *Babesia* förekommer i oväntat hög grad i södra Sverige. Sannolikt sker detta både genom symptomfria infektioner med självläkande förlopp, det vill säga att kroppens eget immunförsvar tar hand om och

bekämpar babesiainfektionen framgångsrikt men sannolikt också med infektioner som inte har fångats av sjukvården och som orsakar mildare eller svårare sjukdom hos individen. Vården bör vara medvetna om denna fästingöverförda smitta och även som smittorisk i samband med blodtransfusioner. Vi har också utvecklat en egen metod för att analysera blodprover för antikroppar mot *Babesia divergens*, som är den sorten som hittills blivit mest uppmärksammad i Europa. Vi har också sett förvånansvärt höga nivåer av antikroppar mot TBEV i Skåne som kan tyda på att även denna fästingöverförda sjukdom skulle kunna vara vanligare än vad vi tidigare trott i denna del av Sverige.

Original papers

Paper I

Svensson J, Hunfeld KP, Persson KEM. High seroprevalence of *Babesia* antibodies among *Borrelia burgdorferi*-infected humans in Sweden. Ticks Tick Borne Diseases. 2019;10:186–90.

Paper II

Svensson J, Christansen CB, Persson KEM, A Serosurvey of Tick-Borne Encephalitis Virus in Sweden: Different Populations and Geographical Locations. 2021. Vector-Borne and Zoonotic Diseases. 21, 8, 614-619.

Paper III

Tijani MK, **Svensson J**, Adlerborn P, Teleka A, Danielsson L, Lövmar M, Forsberg P, Lindgren P-E, Persson KEM. Use of crude *Babesia divergens* merozoite extract in a new ELISA shows high seroprevalence among *Borrelia* infected humans in Sweden. Manuscript.

Author's contribution to the papers

Paper I

J.S. worked with the study samples and the results and wrote the article. J.S., K.P.H., and K.E.M.P. together planned the outline of the study and helped with writing the article.

Paper II

J.S. was responsible for collection of the samples and wrote the article. J.S., C.B.C., and K.E.M.P. together planned the outline of the study and helped with writing the article.

Paper III

M.K.T worked with parasite cultures and laboratory assays together with J.S, L.D. and P.A. M.L and P-E.L. worked with IFA. J.S. performed ELISA assays. J.S. wrote the initital drafts of the article. All authors together planned the outline of the study and helped with writing the article.

Abbreviations

AIRA: Anonymous individuals from rural areas in Skåne

ALT: alanine aminotransferase

AST: aspartate aminotransaminase

ALP: alkaline phosphatase

B: Babesia

B. crassa: Babesia crassa

B. divergens: Babesia divergens

B. duncani: Babesia duncani

B. microti.: Babesia microti

B. venatorum: Babesia venatorum (EU1)

B. bovis: Babesia bovis

B. odocoilei: Babesia odocoilei

B. orientalis: *Babesia orientalis*

B. ovis: Babesia ovis

B. canis: Babesia canis

BBAP: *Borrelia burgdorferi* sensu lato antibody positive group

BBSL: *Borrelia burgdorferi* sensu lato

BBSS: *Borrelia burgdorferi* sensu stricto

CDC: Centers for Disease Control and Prevention (US)

CG: Control group

CRP: C-reactive protein

CSF: Cerebro-spinal fluid

CV: coefficient of variation

ECDC: European Centre for Disease Prevention and Control

ELISA: Enzyme-linked immunosorbent assay

EM: Erythema migrans

ESR: Erythrocyte sedimentation rate

FDA: Food and Drug Administration

FoHM: Folkhälsomyndigheten (The Public Health Agency of Sweden)

IFA: Indirect fluorescent antibody assay

IgG: Immunoglobulin G

IgM: Immunoglobulin M

I. ricinus: Ixodes ricinus.

I. species: Ixodes species.

I. scapularis: Ixodes scapularis

I. persulcatus: Ixodes persulcatus

Hb: hemoglobin

LB: Lyme borreliosis

LD: lactate dehydrogenase

NT: Neutralization test

PCR: Polymerase Chain Reaction

PVM: Parasitophorous vacuole membrane

RBC: red blood cells

iRBC: infected red blood cells

RT: Room temperature

S-KVB: Regional board for quality register

SD: standard deviation

TBD: Tick-borne disease

TBI: Tick-bitten individuals

TBP: Tick-borne pathogens

TBE: Tick-borne encephalitis

TBEV: Tick-borne encephalitis virus

TBEV-Eur: Tick-borne encephalitis virus, European subtype

TBEV-FE: Tick-borne encephalitis virus, Far-Eastern subtype

TBEV-Sib: Tick-borne encephalitis virus, Siberian subtype

TTB: Transfusion transmitted babesiosis

TTI: Transfusion transmitted infection

WB: Western Blot

Introduction to the thesis

Tick-borne diseases (TBD) are diseases which cause considerable disease burden, both in human and veterinarian medicine. They consist of a variety of pathogens such as bacteria, viruses, and protozoa. The transmission is performed by a diversity of tick families. Often the manifestations are varying with unexpected symptoms. In many cases the affected individual hasn't even recalled the incident of the infestation, or maybe just noticed a maybe red or itchy rash. In Sweden some of the TBD have been among the more common reasons to seek medical care for example *Borrelia burgdorferi,* while others have been feared pathogens such as TBEV with potentially life-threating conditions and lifelong disabilities. In some areas the question of vaccination against TBEV has been of big interest and vaccination campaigns founded by the government have been initiated. The question about TBEV as a milder or subclinical illness is to a lesser extent explored.

Globally, the infectious disease malaria caused by the intra-erythrocytic pathogen of *Plasmodium* species causes hundreds of millions of cases of disease each year and hundreds of thousands of lethal cases yearly. The closely related *Babesia* species has in no comparison the same role of affecting humans when it comes to morbidity and mortality. Nevertheless, it does have a lot of similarities in proliferating in vectors, in this case ticks compared with mosquitoes, and it has similar strategies in invading the blood stream and erythrocytes, establishing there, and proliferating and later create lysis of the cell membrane giving rise to parasitaemia with new invasions of erythrocytes and illness in the host. The awareness of the infection, human babesiosis, is established in parts of the US, less is known in Europe and Sweden. The presence of the parasite in Sweden is however well known in veterinary medicine since decades, and sporadic clinical cases have been described in humans but the occurrence of clinical or subclinical cases is at least in part unclear.

Before we started this study, it was assumed that it was mainly animals that was affected by *Babesia*, but since we suspected that also humans could be exposed to the parasites from tick-bites, we wanted to know more about how common it really was in the very southern part of Sweden. Here, both deer and beef are very common, and the climate is milder compared to further north, making it easier for ticks to survive the winters.

To be able to study how common diseases are, easy access to good diagnostic techniques is needed. Further diagnostic and laboratory investigations may clarify additional parts of the pathogen's presence and patterns. Primarily with an approach from a perspective of laboratory medicine, this thesis has the goal of understanding more about two tick-borne pathogens: *Babesia and TBEV: tick-borne pathogens which may be difficult to diagnose.*

Babesia

History and introduction

The parasite *Babesia* was seen and descried the first time more than 100 years ago, and it was named after the Romanian pathologist Victor Babes who discovered the parasites. It was noticed as an intraerythrocytic microbe which then was thought to be a bacterium, in a study of cattle suffering from febrile hematuria. Probably it has actually been described earlier in the literature as a plague affecting cattle in the Bible (Exodus 9:3) (Gelfand JA 1998). A widespread murrain or plague in cattle and other domestic animals was described as "*Behold, the hand of the Lord is upon thy cattle which is in the field, upon the horses, upon the asses, upon the camels, upon the oxen and upon the sheep: there shall be a very grievous murrain".* In Ireland the word "murrain" is still used to describe red-water fever in cattle (Ronald 2004).

During the last century, more and more species of *Babesia* have been discovered, and today we can count to over 100 different ones, and different kind of ticks have been identified as potential transmitters. In 1957 the first human case of Babesiosis was described when a Yugoslavian tailor died, after becoming infected with *Babesia bovis (B. bovis)* (Skrabalo and Deanovic 1957).

Babesia has been described in all inhabited continents and human cases of babesiosis have been diagnosed and confirmed from all continents except Africa, although suspected cases have been seen (Bush, Isaacson et al. 1990) and also signs of serological presence in a larger epidemiological study (Bloch, Kasubi et al. 2018).

Phylogenetics

The phylogenic classification is poorly investigated for *Babesia* species. However, it is a part of the phylum apicomplexan, which also includes *Toxoplasma gondii* and *Plasmodium* (malaria) species, and the order piroplasmida and *Theileria*. *Theileria* multiply in the lymphocytes in contrast to *Babesia* which in the host reproduces in the red blood cells (RBC). *Babesia* can also be transmitted transovarially (Chauvin, Moreau et al. 2009). There is some uncertainty about whether *B. microti* can transmit transovarially (Gray, von Stedingk et al. 2002). This ability of transovarial

transmission has been suggested as one of the factors to differentiate between *B. sensu stricto,* which includes *B. bovis* and *B. ovis* and other human pathogenic species such as *B. microti*-like and another group of Western Clade species including *B. duncani,* where this ability is lacking (Jalovecka, Sojka et al. 2019).

Persistence of transovarial transmission has been demonstrated with an experimental approach with a skin-feeding technique for *B. divergens.* This was confirmed by a positive PCR detection of *B. divergens* DNA performed on pools of various eggs coming from adults infected by skin feeding. (Bonnet, Jouglin et al. 2007)

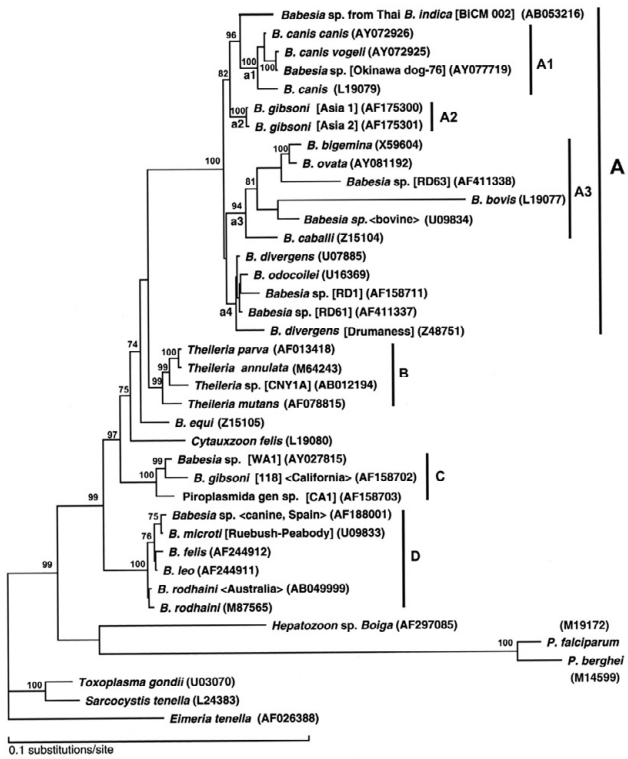


Figure 1: A brief phylogenic approach (Dantrakool, Somboon et al. 2004)

The life cycle

The life cycle of *Babesia* has major similarities with *Plasmodium* spp. (malaria). In contrast to *Plasmodium* though, *Babesia* sporozoites penetrates directly into the RBC and all development stages occur inside the RBC. By binary fission two merozoites are created and this can be done asynchronous. The size and location

differ between different *Babesia* species and host species. In the tick only parasites in a specific pre-gametocyte stage undergo further development and in the ovary of female ticks further transovarial transmission can appear which differ *Babesia* from the closest relative Theileria and most other tick-borne pathogens (TBP). Sporogony takes place in all tick stages and the *Babesia* infection can progress through different stages of tick development, so called transstadial transmission, which is also common for other TBP (Chauvin, Moreau et al. 2009) (figure 2). Longer time of feeding (>36 h) for the tick probably rises the opportunity of establishing in the host (Wilhelmsson, Lovmar et al. 2020).

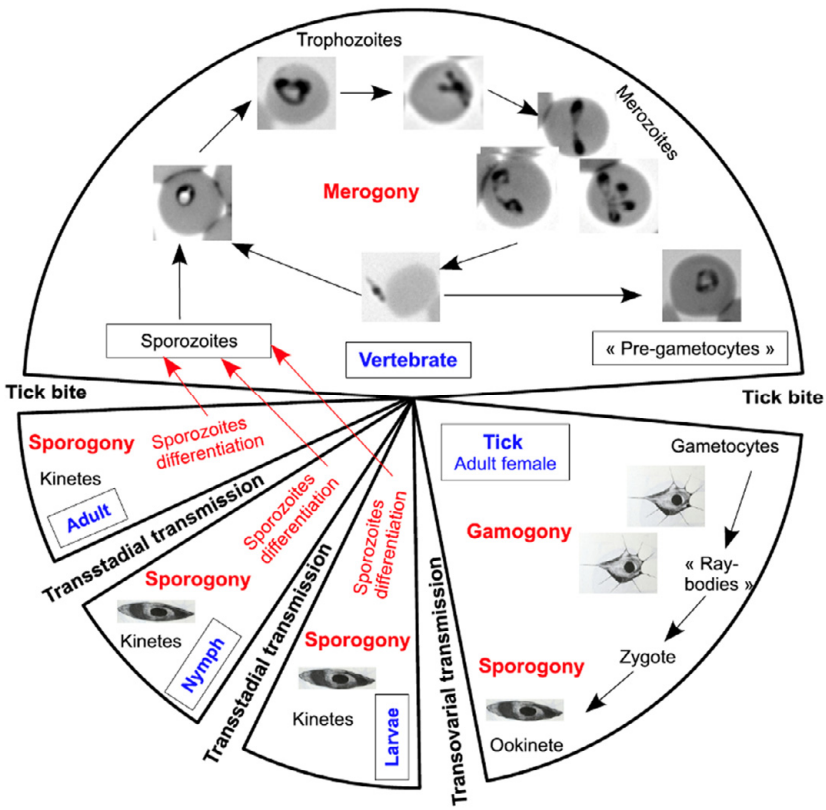


Figure 2: A principal draft of the *Babesia* life cycle. The upper half shows vertebrates, for instance humans. Multiplication is asynchronous and various divisional stages can be seen at the same time in the bloodstream. For instance, anaplasmoid forms that appear just after the penetration of the RBC can be seen together with rounded or ovoid forms (trophozoites) with dividing forms, as well as merozoites which result from the binary fission and these are typically piriform. Gamogony takes place in the tick intestinal cells. Ookinete and kinetes are formed by asexual division. Finally, the differentiation of sporozoites occurs in the salivary glands. Credit, permission and graphic form Chauvin et al and S Wattendorff (Chauvin, Moreau et al. 2009)

Babesia in the red blood cell

Babesia species as well as *Plasmodium* parasites modify the RBC when they infect it. In the iRBC, the modifications both involve membranous structures and proteins as well as intracellular adjustments. This facilitates both the cellular invasion and the intracellular growth. Identifying these specific proteins and molecules has also been suggested as potential targets for drugs and as vaccine candidates (de Koning-Ward, Dixon et al. 2016, Al-Nazal, Low et al. 2022).

As for *Plasmodium* spp., *Babesia* parasites have been seen to adjust the cytoskeleton and influence cytoadhesion for instance in the cerebral course of the infection when sequestered iRBC have been found in cerebral microcapillaries of infected cattle (Hakimi, Yamagishi et al. 2022). Apicomplexan parasites like *Babesia* produce a parasitophorous vacuole membrane (PVM) upon cell invasion, partly by integrating parts of the RBC membrane into the PVM. This protects the parasites from acidification and lysis inside the host cell. When injected into the RBC, *Babesia* spp. seem to break down their PVM more rapidly compared with *Plasmodium* spp*.,* however some membranous structures and transport of vesicles seem to remain (Rudzinska, Trager et al. 1976, Asada, Goto et al. 2012).

For *B. divergens,* some of the best explored proteins/molecules are as follow*s:*

- Apical membrane antigen 1 (AMA1) is involved in host cell invasion by binding to a trypsin- and chymotrypsin-sensitive receptor on the red blood cell (Montero, Rodriguez et al. 2009).
- Glycophorin A and glycophorin B have been shown to likely play a role as entry proteins in RBC invasion (Lobo 2005, Cursino-Santos, Halverson et al. 2014).
- Active serine proteases (p48) and its precursors were recognized to be concentrated in merozoite dense granules in apical secretory organelles involved in erythrocyte invasion. (Montero, Gonzalez et al. 2006, Gonzalez, Estrada et al. 2019).
- Bd37 has been described as a major surface antigen and has been shown to be glycosylphosphatidylinositol (GPI) anchored at the surface of the merozoite (Delbecq 2022, Beri, Singh et al. 2023).

Some proteins have been connected to both *B. divergens* and *B. bovis*, while some are described only in *B. bovis:*

- Spherical body proteins 1-4 (SBP1-4) are involved as signal proteins for *B. bovis, B. divergens* and more (Terkawi, Seuseu et al. 2011).
- VESA1-export associated protein (BbVEAP) seems to be important for parasite growth and potentially for cytoadhesion of *B. bovis, B. divergens* and more (Hakimi, Templeton et al. 2020).

- Variant Erythrocyte Surface Antigen 1 (VESA1) is involved in cytoadhesion for *B. bovis* (O'Connor, Lane et al. 1997).
- The merozoite surface antigen 2 (MSA-2) is a protein of *B. bovis* shown to play a role the RBC invasion (Berens, Brayton et al. 2005).
- Rhoptry-associated protein 1 (RAP-1) is as MSA-2 also seen to be expressed in *B. bovis*. and involved in RBC attachment (Mosqueda, McElwain et al. 2002).

Most of the studies relate to *B. bovis* and *B. divergens* but the overlap between different *B.* species seems to be widespread. *B. microti* has not the same properties concerning cultivation possibility. Many of the proteins have been connected to vaccine candidates and therapy candidates as well as therapy resistance (Hakimi, Yamagishi et al. 2022). A recent study has also showed modulation of erythrocytic metabolites of all classes as lipids, amino acids, carbohydrates, and nucleotides that can benefit parasite growth of different metabolic components. This can also open possibilities of new therapeutic targets (Beri, Singh et al. 2023).

Animals

From a European perspective, babesiosis has been well known as a veterinarian disease for a long time. Especially among cattle it has been diagnosed and treated during summer. In colloquial language it is called “the summer disease” (Sw: sommarsjuka) or “blood peeing” (Sw: blodpinkning, blodhalning) (Karlsson and Andersson 2016). Among cattle in different parts of Sweden where some of the animals had shown symptoms of babesiosis, a study revealed 53% of the animals to be positive in a molecular biological investigation with polymerase chain reaction (PCR) for *B. divergens* (Andersson, Vichova et al. 2017). Other animals such as roe deer are also carriers of the parasite, and in one investigation 52% were positive with mainly *B. venatorum* (Andersson, Bergvall et al. 2016).

Ticks

The clinical picture of human babesiosis has many similarities with the other intraerythrocytic parasitemia, malaria. The peak incidence of TBD correlates with the tick bite season which in Sweden is from around May to September, even if activity among *Ixodes ricinus (I. ricinus),* which is the completely dominating tick in Sweden, has been noticed all winter in southern parts of Sweden. In fact, most individuals don’t remember the tick infestation (Hildebrandt, Gray et al. 2013).

I. ricinus is considered to be the main vector in Europe for *B. divergens, B. microti* and *B. venatorum.* A study in Sweden where *I. ricinus* ticks were investigated found that the ticks were seen to carry (in total 4% of them) both *B. divergens* and *B. venatorum,* but mostly *B. microti*, which is a bit surprisingly since the latter one has mostly been considered to be a pathogen present in North America (Karlsson and Andersson 2016). Similar percentages have been detected for *B. microti* in Poland among *I. ricinus* (Wojcik-Fatla, Cisak et al. 2006) while also another species, *Dermacentor reticulatus,* have been noticed to carry *B. microti* with similar percentages (Wojcik-Fatla, Bartosik et al. 2012).

In infected ticks, *Babesia* parasites appear in the salivary glands. The ticks are not infectious until they have undergone development, which happens when the tick starts to feed. It can take about two days to complete a feeding (Joyner, Davies et al. 1963, Hildebrandt, Zintl et al. 2021). After tick attachment to the host, it could be up to 24 to 48 hours before the infection is transmitted, at least for *B. microti.* (Belman 1999, Pujalte and Chua 2013).

For *B. venatorum,* the roe deer has been proposed to be a principal reservoir while cattle have been pointed out for *B. divergens*. *I. ricinus* also serves as a reservoir host since *B. divergens* has been shown to be transmitted both transstadial and transovarial (Gray, Zintl et al. 2010, Krause 2019). The number of *I. ricinus* ticks and the geographical spread in Sweden have both been seen to increase considerably during the latest decades, which has increased the focus on tick borne pathogens (Jaenson, Jaenson et al. 2012). Previously non-infested areas have been subject to Ixodidae ticks establishing and climate changes with mountainous or more subarctic climate have been seen new changing with milder winters, longer summers and increasing number of Ixodidae ticks (Jaenson, Talleklint et al. 1994, Talleklint and Jaenson 1998, Lindgren, Talleklint et al. 2000, Medlock, Hansford et al. 2013).

In comparison with other continents, *Ixodes scapularis (I. scapularis)* has been shown to be the primary tick vector in Eastern US for *B. microti,* and small rodents such as mice and voles are the primary reservoir hosts. An increase in the number of white-tailed deer is also thought to help increase the spread (Spielman, Wilson et al. 1985, Krause 2019). In Western US and Canada the spread of *B. duncani* is mainly considered to originate from *Dermacentor albipictus* (Scott and Scott 2018). In Eastern Asia, *Ixodes persulcatus (I. persulcatus)* has been considered as the main transmitter of *B. crassa., B. microti* and *B. venatorum. I. persulcatus* has also recently been found in Northern Sweden, probably spreading from the east with the potential of bringing new tick-borne pathogens into Sweden (Jaenson and Wilhelmsson 2019, Kjaer, Soleng et al. 2019) (table 1).

Table 1: Suggested vectors for *Babesia* species with human pathogenicity in different continents. Modified from different references (Jaenson and Wilhelmsson 2019, Krause 2019, Hoffman, Olsen et al. 2023)

***Babesia* species:**	**Vector, probable or confirmed:**
Asia	
B. crassa-like	*Ixodes persulcatus, Haemaphysalis concinna*
B. microti	*Ixodes persulcatus, Ixodes ovatus*
B. venatorum	*Ixodes persulcatus*
Europe	
B. divergens	*Ixodes ricinus*
B. microti	*Ixodes ricinus, Ixodes persulcatus*
B. venatorum	*Ixodes ricinus*
United States	
B. microti	*Ixodes scapularis*
B. duncani	*Dermacentor albipictus*
B. divergens-like	*Ixodes species*

Human Babesiosis, seroprevalence and zoonotic aspects in different parts of the world

Sweden: case descriptions

When it comes to cases of human babesiosis in Sweden, only a few and sporadic cases have been described and published. In Uppsala in 1992 it was described how a healthy but splenectomized 34-year-old man rapidly developed severe disease with multiple organ failure, and he required intensive care treatment before adequate diagnose and treatment. A massive parasitemia (>40% in percentage of parasitized erythrocytes with *B. divergens* was seen in blood smears (Uhnoo, Cars et al. 1992). After that, sporadic cases have been diagnosed in other parts of the country such as Göteborg and Halmstad (unpublished cases).

In Lund in 2015 a 52-year-old splenectomized patient with previous leukemia (including earlier cytostatic treatment) experienced repeated periods of fever and myalgia. Despite broad spectrum treatment against bacteria, fungi, and viruses no improvement was obtained. Laboratory pathologies were leukocytosis, anemia, signs of hemolysis (high lactate dehydrogenase and low haptoglobin), signs of inflammation including high C-reactive protein (CRP) and ferritin and impact of functions of liver and kidney with elevated aspartate aminotransferase, AST, and creatinine. Since the awareness for the parasite wasn't present, the diagnose was delayed until an observant laboratory technician by accident discovered *B.*

venatorum in a blood smear. Retrospective examination of blood and bone marrow smears done because of unclear fever revealed that parasitemia had existed for several months without being discovered (Bläckberg, Lazarevic et al. 2018). The overall situation and lack of awareness of the pathogen has also been discussed in a national report for medical professionals (Svensson, Lazarevic et al. 2019).

Europe: case descriptions

After the first described case in 1957 in the current Croatia (Skrabalo and Deanovic 1957) about 50-100 cases have been seen in different parts of Europe according to European Centre for Disease Prevention and Control (Hildebrandt, Gray et al. 2013, ECDC 2020). Most cases have been noticed in France (Martinot, Zadeh et al. 2011) and the British Isles (Kennedy 1980, Browne, Ryan et al. 2010) but also in widespread parts of the continent as Austria (Herwaldt, Caccio et al. 2003), Finland (Haapasalo, Suomalainen et al. 2010), Germany (Haselbarth, Tenter et al. 2007), Italy (Herwaldt, Caccio et al. 2003), Montenegro (Andric, Golubovic et al. 2012), Portugal (Centeno-Lima, do Rosario et al. 2003), Poland (Welc-Faleciak, Hildebrandt et al. 2010, Moniuszko-Malinowska, Swiecicka et al. 2016), Spain (Asensi, Gonzalez et al. 2018) and also suspected imported cases to Czech Republic (Nohynkova, Kubek et al. 2003) and Switzerland (Baumann, Pusterla et al. 2003). Many of the cases are due to *B. divergens* but also *B. venatorum*, *B. microti* and *Babesia odocoilei (B. odocoilei)* occur.

Serology in Europe

Since human babesiosis in many cases can be self-limiting or misdiagnosed, different studies of seroprevalence have been performed in different parts of Europe. In Southern Norway a large study of about 1,500 adults showed that 2.1% had antibodies against *B. microti* (Thortveit, Aase et al. 2020). In Mid-western Germany tick-exposed people were investigated and among people with Lyme borreliosis (LB), 11.5% had antibodies when an indirect fluorescent antibody test (IFA) against *Babesia* was used, with more individuals having antibodies against *B. microti* compared to *B. divergens.* (Hunfeld, Lambert et al. 2002).

In Poland and Italy people with professions with higher risk for tick-borne diseases have been investigated and in Poland 4.4% of foresters had IgG directed against *B. microti* and in Italy about 19% in total had antibodies against either *B. microti* or *B. divergens* but also *Babesia canis (B. canis)* and *Babesia bovis* (*B. bovis*) (Pancewicz, Moniuszko et al. 2011, Chmielewska-Badora, Moniuszko et al. 2012, Gabrielli, Calderini et al. 2014). In a study in Belgium, 199 patients previously treated for tick-borne diseases about 40% were seropositive for *B. venatorum* while

33% where seropositive for *B. divergens* and 9% for *B. microti* (Lempereur, Shiels et al. 2015).

A recent review concluded that except for described cases of human babesiosis that have occurred in most countries, serology studies have nowadays disclosed presence of many different *Babesia* spp. in many parts of Europe and an increasing presence is expected both in human and veterinarian medicine (Bajer, Beck et al. 2022) .

In summary, we can conclude that the seroprevalence varies but in many cases is surprisingly high, both in actual percentages as in Belgium (Lempereur, Shiels et al. 2015) and Germany (Hunfeld, Lambert et al. 2002), but also relatively high findings of *B. microti.* Mostly the investigations have used different types of IFA protocols.

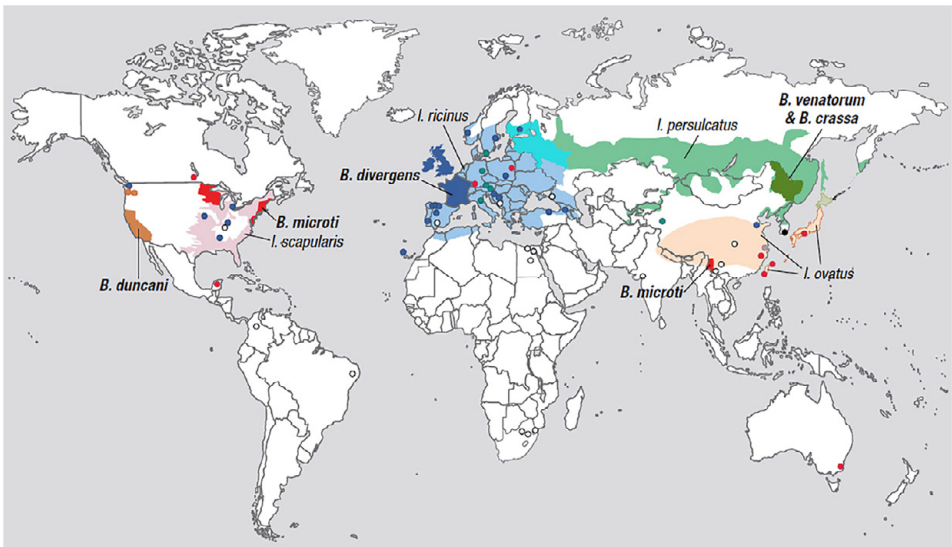


Figure 3: Worldwide distribution of human babesiosis and Ixodes tick vectors. Dark colors indicate areas where human babesiosis is endemic or sporadic (defined by >5 cases). Light colors indicate areas where tick vectors are present but human babesiosis is rare (<5 cases), undocumented, or absent. Credit: Vannier E., Krause P.J. 2019. Babesiosis.

North America

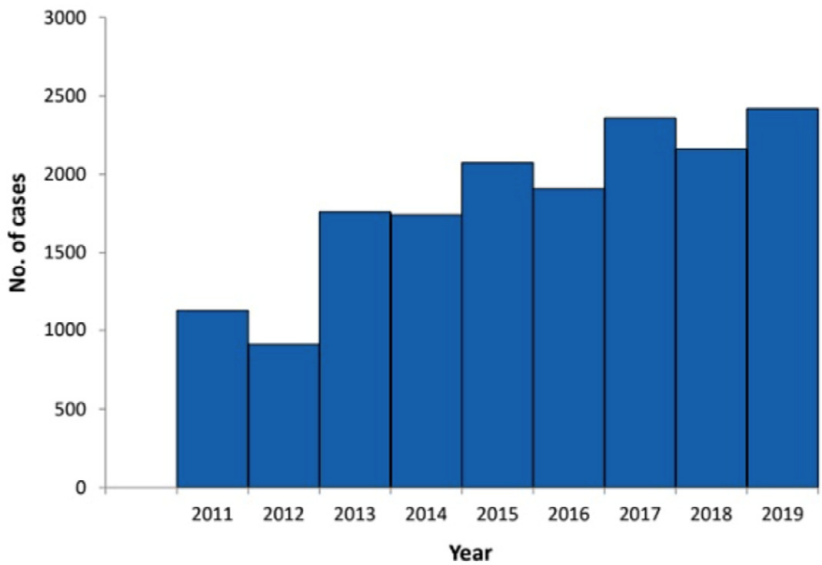
Human babesiosis is considered to be a zoonotic disease. As mentioned, it's been discovered all over the world. In the US, *B. microti* has had a lot of attention and the cases of human babesiosis is today more than 2,000 cases per year according to Centers for Disease Control and Prevention (CDC 2019) (table 2) A few states have most of the human babesiosis cases in the Northeast and upper Midwest, especially in parts of New England, New York state, New Jersey, Wisconsin, and Minnesota.

There is a seasonal peak seen between May and October. Rodents and cattle act as zoonotic reservoirs while *Ixodes* species are vectors. In Western US, cases of human babesiosis have mainly been seen to be caused by *B. duncani* (Swei, O'Connor et al. 2019).

Factors contributing to the increasing numbers during the last decades are increased awareness among health care professionals, public awareness, diagnostic opportunities but also ecologic aspects like mice spreading in combination with the spreading of the co-infection *Borrelia burgdorferi* since these tick-borne pathogens seem to have synergistic effects for proliferation (Dunn, Krause et al. 2014, Diuk-Wasser, Vannier et al. 2016).

In total 20,000 cases have been notified in the US while in Canada more than 1,000 cases have reported with an overweight for the western parts of the country and mainly caused by *B. duncani* (Kumar, O'Bryan et al. 2021, Yang, Christie et al. 2021),

Table 2*:* Number of reported cases of human babesiosis, by year 2011-2019 in the US. Notable is that in many diagnoses there is a discrepancy between the number of reported cases and the actual number of cases in the healthcare system. Credit: CDC, updated 2021/11/04



Other parts of the world

A region in Northwestern China has been found to be endemic for *B. venatorum* and also cases of *B. crassa*-like parasites have been found (Jiang, Zheng et al. 2015, Jia, Zheng et al. 2018). China is considered to have the highest number of reported cases of human babesiosis outside North America. *Babesia* species in other continents include *B. crassa*-like, *B. divergens, B. microti* and *B. venatorum*, which have all been detected. Phylogenic similarity has been seen when compared to strains in Europe but also in North America. Interestingly, presence of *B. microti* has been seen in areas with malaria in Southwestern China and coinfections with *Plasmodium* species and *B. microti* have been reported (Zhou, Li et al. 2013, Kumar, O'Bryan et al. 2021).

In Japan, cases of human babesiosis with *B. microti* have been reported including a case with blood transfusion transmitted disease. In the *Ixodes ovatus*, the tick vector in Japan and in wild rodents' high presence of molecular detection have been done with prevalence's of between 13% and 45% (Hussain, Hussain et al. 2021, Kumar, O'Bryan et al. 2021).

Other cases of *B. microti* have been reported from all populated continents in countries such as Australia and Mexico, and cases with human babesiosis from unidentified *B.* spp. have been found in Cuba, Egypt, India, Taiwan, South Africa and Colombia (Vannier and Krause 2012, Krause 2019, ECDC 2020).

Other routes of transmission: transfusion and others

Except for the prime route of transmission through ticks, there are also other ways. One critic way of transmission is through blood products and in the US a couple of hundred cases have been registered. Some of these have also had a fatal outcome (4%) (Linden, Prusinski et al. 2018). Human babesiosis is now considered as the transfusion transmitted infection (TTI) with one of highest transfusion related mortality according to the American Food and Drug Administration (FDA 2018) (Ellingson 2017). Screening for this TTI has now been recommended in the *B.* species. endemic parts of the US and may be recommended in greater parts of the country, and it has also been shown to reduce the risk for human babesiosis among recipients.

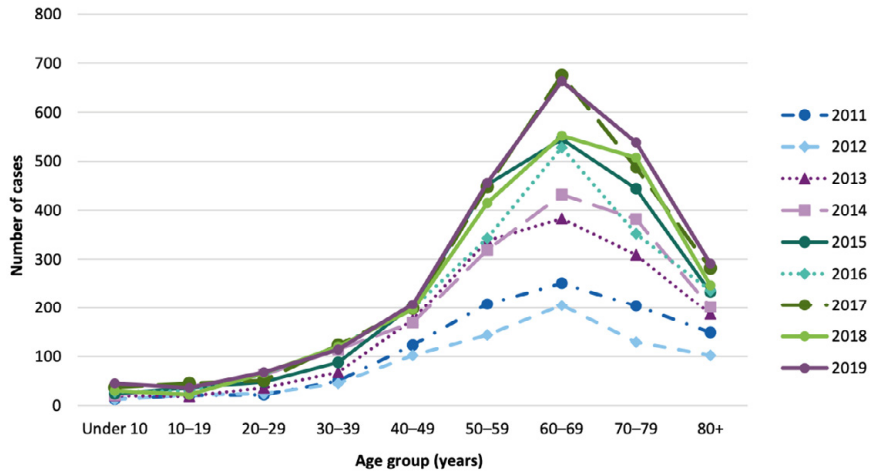
In a large study of about 90,000 blood donation samples tested, 29 cases of transfusion transmitted babesiosis (TTB) could be linked to *B. microti*-infected donors (Moritz, Winton et al. 2016, AABB 2017, FDA 2018). In another summary, also some cases of *B. duncani* and *B. divergens*-like (MO-1) have been described to

cause TTB even if *B. microti* today seems as the most common babesia-related TTI in the US (Tang and Tran 2020).

The role of different blood group systems has been pinpointed in this infection as well as for other pathogens. In malaria, the ABO system has been investigated and that's also the case for *B. microti.* In one study especially RhD+ blood group is mentioned as a risk factor but also the ABO system could be a correlating risk factor for an infection to establish (Jajosky, O'Bryan et al. 2023). However it is clear that the parasites change the RBC, for instance by modifying protein expression and cellular membrane rearrangement, when invasion takes place and connected to immune evasion, cytoadhesion, and nutrient uptake (Hakimi, Yamagishi et al. 2022). More seldomly transmission has been found to be transplacental, perinatal or in conjunction with transplantation of solid organs (Hildebrandt, Gray et al. 2013, Brennan, Herwaldt et al. 2016).

Clinic

All ages are affected by human babesiosis but there seems to be a concentration of middle-aged individuals around 50-70 years (figure 4). More severe disease often occurs in elderly patients. Other prominent risk factors are disturbed and/or reduced splenic function, most commonly in connection with a previous splenectomy in the medical history, or depressed cellular immunity (Hildebrandt, Gray et al. 2013). The symptoms of babesiosis are often general and non-specific with fever and with flu-like symptoms such as headache, chills, sweats, and myalgia. In some immunocompetent patients the symptoms may persist for weeks and even months and some have eventually demanded pharmacological treatment. In these more serious clinical stages and in immunocompromised patients, considerable hemolysis may develop followed by anemia, jaundice, dark urine, kidney failure and also effect on other organs such as acute respiratory distress syndrome (Collet, Thiele et al.), heart failure, coagulopathy and disseminated intravascular coagulation (DIC) and also involvement of other organs as CNS (Vannier and Krause 2012, Hildebrandt, Gray et al. 2013).



* Data on age were available for most case-patients (2011, n = 1,041/1,126; 2012, n = 785/911; 2013, n = 1,523/1,761; 2014, n = 1,740/1,742; 2015, n = 2,074/2,074; 2016, n = 1,902/1,909; 2017, n = 2,347/2,358; 2018, n=2,157/2,161; 2019, n=2,418).
† Year as reported by the health department.

Figure 4: Chart showing the age distribution of human babesiosis. Age should be taken into account as a risk factor. Credit: CDC, updated 2023/01/05.

Diagnosis

Clinical chemistry and haematology

Laboratory testing can contribute with guidance and support in diagnosing and following the cause of human babesiosis. Elevated liver enzymes such as transaminases (ALT, AST) and ALP, LD can be seen as well as unconjugated bilirubin and lowered haptoglobin with a pattern indicating intravascular hemolysis (Hildebrandt, Gray et al. 2013). ALT and AST have also been proposed as possible screening analytes for *B. microti* (Primus, Akoolo et al. 2018). Urinary analysis may reveal hemoglobinuria, hematuria and an excess of urobilinogen, and in serum renal markers such as creatinine and cystatin C may raise as a sign of renal failure (Krause 2019).

When it comes to hematology, auto-analyses of blood smears have repeatedly failed to detect intracellular inclusions and direct questioning and/or conscious laboratory staff are required (Bruckner, Garcia et al. 1985, Bläckberg, Lazarevic et al. 2018). Other hematology parameters are anemia with a pattern of normochromic and normocytic anemia, and there can also be thrombocytopenia and occasionally

leucopenia as well, and a positive Combs test can also be indicative for human babesiosis (Hildebrandt, Gray et al. 2013).

Classic clinical inflammatory signs in plasma of human babesiosis includes elevated C-reactive protein (CRP) but also other signs like elevated ferritin and erythrocyte sedimentation rate (ESR) have been described. In dogs infected with *B. canis,* a more extensive study of inflammatory markers with serum protein electrophoresis patterns showed classic fluctuations with hypoalbuminemia and elevations of also other acute-phase reaction proteins (Bläckberg, Lazarevic et al. 2018, Asawakarn and Taweethavonsawat 2021).

From a hematological point of view it's also notable with observations of development of the rare but dangerous condition of hemophagocytic lymphohistocytosis, which has been linked to *B. divergens, B. microti* and *B. venatorum* (Miguelez, Linares Feria et al. 1996, Poisnel, Ebbo et al. 2013, Gonzalez, Castro et al. 2015, Bläckberg, Lazarevic et al. 2018)

Direct detection

The traditional method of detecting *Babesia* parasites is to review Giemsa-stained blood smears. Inside the RBC, ring forms or piriform inclusions can be seen with light blue cytoplasm. In a mild or moderate disease as well as early in the course of human babesiosis a low level of parasitemia can appear but might be difficult to visualize. Repeated blood smears can be of value. The most important differential diagnosis is malaria. *Plasmodium* parasites can present pigment (hemozoin, derived from hemoglobin) and *B.* spp. can often form tetrads, "Maltese cross" (figure 5). The morphology between different *B.* spp. merozoites can differ but it is uncertain to decide with this technique and instead other diagnostic tools should be added (Mylonakis 2001, Hunfeld, Hildebrandt et al. 2008, Hildebrandt, Gray et al. 2013).

Polymerase chain reaction (PCR) is an alternative for direct detection. For instance, a method targeting the 18S rRNA gene of *B. microti* is considered to be very sensitive in detecting as little as 5 parasites/µmol and it can also detect other *B.* spp. Other methods such as real time PCR-assays have also been developed. It's also important to notice that reversion of PCR-results may lag after treatment of human babesiosis, since molecular fragments may persist in the circulation (Teal, Habura et al. 2012, Hildebrandt, Gray et al. 2013).

From a research point of view an alternative is also inoculation of whole blood into for example golden hamsters, jirds, sheep or mice (Malandrin, L'Hostis et al. 2004, Hunfeld, Hildebrandt et al. 2008).

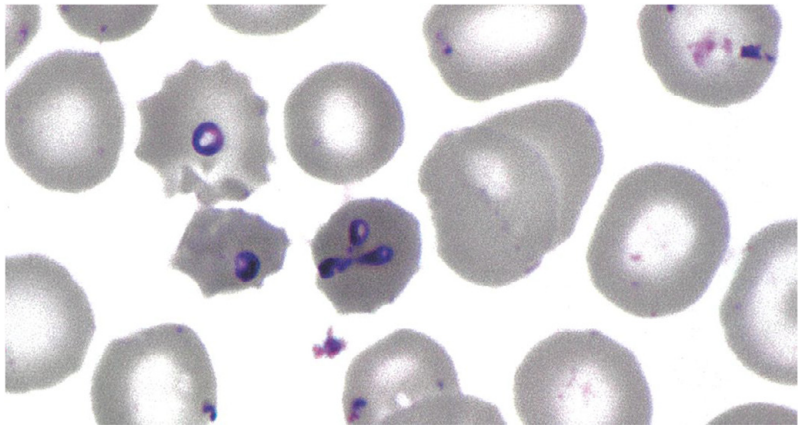


Figure 5: *B. venatorum* inside RBC. Credit K E M Persson

Indirect detection

Serology tests are important for supporting and confirming the diagnosis. To ascertain an acute or convalescent IgG titer a repeated analysis can be required to find significant differences and to distinguish a recent infection from a past infection. Acute phase serology tests have limited value but for instance in illness with *B. microti* IgG titers usually exceed 1:1024, but typically decline to 1:64 or less within one year (Levin, Williamson et al. 2016) Notable is also that a cross reactivity exists between *B. divergens* and *B. venatorum* tests. (Vannier, Diuk-Wasser et al. 2015).

When it comes to antibody detection techniques indirect fluorescent antibody (IFA) test and enzyme-linked immunosorbent assay (ELISA) are the most used. One IFA for *B. microti* have been tested in various laboratories throughout the US and been held as possible standard method even if the positive predictive value (PPV) is fluctuating between different laboratories (from 69% to 100%) (Krause, Telford et al. 1994). Analysis of IgM is considered to be less specific compared with IgG. It is also important to consider cross reactivity against closely related members of Apicomplexa as *Plasmodium* spp. and *Toxoplasma* (Hunfeld, Lambert et al. 2002).

An alternative for various *B.* spp. but at least *B. divergens* among human pathogenic variants is ELISA. In can be used with easier handling, it is more time effective and reliable when standardized compared to IFA. Also compared to IFA, it can be seen as less subjective in the analytical process without individual assessment in examining the glasses. The right blocking conditions and purification procedures of antigen which are required in a higher amount than for IFA is of importance. The antigens which are used are often merozoite surface proteins or rhoptry-associated proteins (Hildebrandt, Gray et al. 2013). A prototype immunoassay has also been

developed to be analyzed on Abbott Architect multichannel automated instrument for *B. microti* (Cheng, Coller et al. 2018).

Treatment

The knowledge about treatment is mainly based on *B. divergens* and *B. microti.* Combination treatment with antibiotics together with antiparasitic medications are common. The substances atovaquone or quinine and azithromycin or clindamycin are the most common options. In milder cases of *B. microti,* atovaquone and azithromycin are the most commonly recommended combination but in severe cases of *B. microti* and when human babesiosis is caused of *B. divergens, B. duncani,* or *B.* venatorum the combination of clindamycin and quinine is recommended. The duration of treatment varies but shorter regimes of 7-10 days often need to be extended up to 6 weeks or more. Immunocompromised patients and severe cases may require blood exchange transfusion and hemodialysis.

Factors that indicate severe human babesiosis include parasitemia (>10% iRBC), severe hemolysis and anemia (Hb <100g/L), pulmonary, renal, or liver impairment. Other antimicrobial, antimalarial, and antiprotozoal drugs may be alternatives in severe and special cases (Hildebrandt, Gray et al. 2013, Saifee, Krause et al. 2016, Krause 2019). There are resistance issues in the treatments which strengthen the need of a combination regime. It is also important to monitor the treatment and different parameters as grade of parasitemia, organ function and biochemical analytes to rapidly discover parasite relapse (Ord and Lobo 2015).

It has been shown that there is an increased risk with absence of adequate treatment. Human babesiosis may persist for months or even years. The combination of clindamycin and quinine is far from a perfect regime and the may persist and develop periods of new symptoms long time after the start of the infection (Krause, Spielman et al. 1998).

Vaccination

The question of vaccination has been a topic both in human and veterinary medicine. Attempts have at least been tried out for a vaccine against *B. microti*, *B. bovis*, *B. bigemina*, *B. orientalis* and *B. divergens*. Different strategies have been applied. For instance, an inactivated/killed and attenuated/weakened whole-parasite vaccine against *B. microti* has been used. Whole-parasite vaccines have not managed to show convincing efficacy against *B. microti* and the presence of RBC membranes in whole-parasite vaccines is a risk of inducing antibodies against the recipient's RBC (Al-Nazal, Cooper et al. 2021, Al-Nazal, Low et al. 2022, Jerzak, Gandurski

et al. 2023). Vaccine attempts on cattle has also resulted in fatal cases. Attempts with vaccines targeted against subunits have also been investigated. For instance, *B. microti* surface antigen and various other *B. microti* proteins have been tried out as targets. Even though there is diversity for the different suggested proteins this may be a more promising way. The opportunity of cultivating *B. divergens* in vitro opened up opportunities to produce a whole-parasite vaccine in the future (Rodriguez, Alhassan et al. 2014, Al-Nazal, Low et al. 2022, Jerzak, Gandurski et al. 2023).

Prognosis

The prognosis of human babesiosis is dependent of the general physical condition and age. High age has been shown to be a risk factor for severe disease and fatal course. Other distinct risk factors are impaired splenic function, HIV infection and immunocompromised patients, for instance several patients have been seen with hematological malignancies and ongoing chemotherapy (Vannier, Diuk-Wasser et al. 2015). When it comes to the immune defense the cellular immune response is considered to be important for protecting and healing human babesiosis completely, including the splenic function and mediators such as interleukins (IL) 12 and 18 as well as tumor necrosis factor (TNF) α and interferon γ. The humoral immune response is considered to have an important assistant role in keeping the parasitemia low but lacks ability to clear the infection completely. It has been suggested in bovine vaccine models that mediation occurs with cytotoxic activity (IgG1), opsonization (IgG2), and complement activation (Chauvin, Moreau et al. 2009).

In the US about 50% of the human babesiosis patients need inpatient care with a median time in hospital of 4 days (CDC 2019). The overall mortality is considered to be around 0.5-1% but significantly higher among TTB (about 4%) which might be connected to older and more fragile patients receiving blood transfusions (Menis, Forshee et al. 2015, Vannier, Diuk-Wasser et al. 2015, Linden, Prusinski et al. 2018).

Borrelia Burgdorferi – in comparison with *Babesia*

Borrelia burgdorferi sensu lato (BBSL) is a tick-borne pathogen that manifests as Lyme borreliosis (LB). LB is the most frequent tick-borne disease (TBD) in Sweden and in many other countries. The annual incidence in South-eastern Sweden among humans is 0.5% (Bennet, Halling et al. 2006). Two studies of *I. ricinus* ticks have shown that about 20% of the ticks (above 30% in adult ticks, 15% in nymphs) are

infected with BBSL (Wilhelmsson, Fryland et al. 2010, Wilhelmsson, Lindblom et al. 2013). Interestingly, one study has shown that ticks which already are infested with BBSL easier gain a co-infection with *B. microti* (Dunn, Krause et al. 2014). Except for *I. ricinus* ticks in Europe, *I. scapularis* and *Ixodes pacificus* are prime transmitters in North America, and in Russia and Northern Asia *I. persulcatus* is the main transmitting pathogen.

In the US presence of coinfection with BBSL and *B. microti* have been stated to be up to 13% in *Ixodes* spp. and up to 41% in rodents (Diuk-Wasser, Vannier et al. 2016). In comparison between *Babesia* species and BBSL both pathogens have the ability to transmit transstadially, remaining in the vector from one life stage to the next while some *B.* spp. also has the possibility to transmit transovarially in certain arthropod vectors from parent arthropod to offspring arthropod with the pathogen (Chauvin, Moreau et al. 2009).

The clinical picture is partly overlapping between the two pathogens. A classic symptom for LB is erythema migrans (EM). LB and human babesiosis as coinfection probably give a more severe and prolonged disease. LB is well known for potential neurological disease as well as arthralgia. The standard treatment for the well-known LB is also having a week effect on human babesiosis which could result in a concealed human babesiosis where diagnosis is missed or delayed (Diuk-Wasser, Vannier et al. 2016). It is also suggested that parallel *B. microti* infection should be considered when moderate to severe LB has been diagnosed. Coinfection could mean increased severity and duration of the LB clinical development (Krause, Telford et al. 1996).

Tick-Borne Encephalitis Virus

The pathogen

Tick-borne encephalitis (Haglund, Settergren et al.) has been noticed more and more during the 20th century. It was described as a disease in 1931 and the pathogen, tick-borne encephalitis virus (TBEV) was described in far-eastern Russia by Zilber in 1937. TBEV belongs to the genus *Flavivirus*, consisting of three major different subtypes: European (TBEV-Eur), Siberian (TBEV-Sib) and Far-Eastern (TBEV-FE) (Charrel, Attoui et al. 2004). More subtypes have been suggested later on and taxonomies with five or seven subtypes have been described (Kovalev and Mukhacheva 2017, Deviatkin, Karganova et al. 2020).

The clinical feature of TBE consists of a biphasic pattern, especially for TBEV-Eur, beginning with the first phase of nonspecific symptoms such as mild fever, myalgia, nausea, and headache. In some cases, a second phase may follow one to two weeks later. The second phase affects the central nervous system with potentially severe neurological symptoms due to meningitis or encephalitis. There is no pathogen specific treatment offered but only general health care interventions can be used (Charrel, Attoui et al. 2004).

In Sweden only the TBEV-Eur has been found so far. The primary vector is the *I. ricinus* tick, even though the primary vector of TBEV-Sib, *I. persulcatus* has been found in the northern part of Sweden (Jaenson, Jaenson et al. 2012, Jaenson and Wilhelmsson 2019, Kjaer, Soleng et al. 2019). The first case in Sweden was described in the 1950s and the disease was in the beginning mostly noticed in the archipelago and coastal areas of Stockholm. During the latest decades the *I. ricinus* has been reported from larger and larger parts of Sweden (Jaenson, Jaenson et al. 2012) and consecutively the TBE cases have been more and more present in many parts of southern and central parts of Sweden (Jaenson, Hjertqvist et al. 2012).

Epidemiology

Factors such as migratory birds and roe deer's as reservoir animals have been proposed as important factors in spreading ticks and TBD including TBEV, and the quantity of *I. ricinus* has increased in the Region of Skåne (southernmost Sweden)

lately (Jaenson, Jaenson et al. 2012). Also in Skåne an increasing number of cases of TBE have been seen both in various parts of the county but also in actual numbers and the last years between 5 to 10 clinical cases have been diagnosed annually (Fält, Alsterlund et al. 2006, Waldeck 2019). Among people with a history of tick-bites and suspected TBD there has also been descriptions of subclinical or mild infections of TBEV in the same area (Fält, Alsterlund et al. 2006, Lindblom, Wilhelmsson et al. 2014). This has also been seen in the neighboring region of Blekinge (Stjernberg, Holmkvist et al. 2008). In the Åland islands, located between Sweden and Finland, which is a high risk area of TBD, a study showed 5% of blood donors to have IgG antibodies against TBE, hence subclinical TBE cases are considered as common (Wahlberg, Carlsson et al. 2006).

TBEV hot spots or foci areas have been seen to be very concentrated, and in limited geographical areas relatively many cases can appear. The pattern has been seen for instance in Germany and Denmark (Agergaard, Rosenstierne et al. 2019, Lang, Chitimia-Dobler et al. 2022) and the pattern has also been recognized in southern Sweden (Waldeck 2019).

In other parts of the world, TBEV-FE and TBEV-Sib strains causing more severe forms of the TBE (Belikov, Kondratov et al. 2014, Ruzek, Avsic Zupanc et al. 2019).

Diagnosis

Except for the symptoms, laboratory features can be abnormal biochemistry analyses such as leucocytosis, elevated ESR and CRP. Also signs in cerebrospinal fluid (CSF) such as monocytosis and signs of impairment of the blood-cerebrospinal fluid barrier (Charrel, Attoui et al. 2004) can occur.

When it comes to serological assays for TBEV, different IgG and IgM assays have been evaluated during many years. Specificity is seen as a problem in all cases (Ackermann-Gaumann, Tritten et al. 2018). A possibility is to use a neutralization test (NT) which can differ between vaccination serological responses and true infection responses, since true infections induce a response against both whole virus and non-structural protein 1, whereas the vaccination does not result in antibodies against the latter one (Albinsson, Vene et al. 2018).

In various countries in Europe a retrospective study indicated suspected cases of TBE also with neurological effects when investigating sera and CSF. But also, the fact that people have received vaccinations for TBEV and other flaviviruses, where a known existing cross-reactivity is known, affect the serological findings (Haglund, Settergren et al. 2003). Since the flaviviruses are small and by parts have overlapping structural proteins between them, cross-reactivity of serological investigations appear (Heinz and Stiasny 2012).

Other tick-borne pathogens

Other pathogens

In Sweden and northern Europe other tick-borne pathogens causing TBD are *Rickettsia* spp. (spotted fever group), *Anaplasma phagocytophilum* and *Neoehrlichia mikurensis*. *Rickettsia* spp. can usually cause a mild fever but in some cases severe disease with myocarditis and meningitis has been seen. *Anaplasma phagocytophilum* is if symptomatic usually causing a mild fever and flu-like disease but can in severe cases cause organ failure including coagulopathies and kidney failure (Lindblom, Wallmenius et al. 2013, Dunaj, Moniuszko-Malinowska et al. 2018, Ocias, Wilhelmsson et al. 2020). *Neoehrlichia mikurensis* has been found in several European countries but has only in rare cases been described to cause severe disease in immunocompromised patients with fever and flu-like disease (Jenkins and Kristiansen 2013). It has also been found in some clinical cases in Sweden, as well as cases of infected blood donors have been seen with PCR confirmation (Grankvist, Andersson et al. 2014, Labbe Sandelin, Olofsson et al. 2022).

The incidence of tick-borne co-infections

As previously described co-infections occur between *B.* spp. and BBSL and are described to facilitate each other in spreading. It seems like there are ecological and zoonotic factors that can be beneficial for *B. microti* and BBSL to spread and proliferate together. For instance, factors such as tick abundance and tick feeding behaviour can affect tick–host contact rates. The establishment of BBSL in an area seem to increase the possibility of establishment of *B. microti*. (Dunn, Krause et al. 2014, Diuk-Wasser, Vannier et al. 2016). Especially in the US this co-incidence has been described. Serological data shows a large overlap with about 20% of LB patients also having *Babesia* antibodies, while also 25% of human babesiosis patients have been shown to have BBSL antibodies. It is plausible that some treatments for LB can have some effect of an active human infection with *Babesia*. Anyway, it's described that human babesiosis in some cases can be a difficult infection to eliminate and that an extended treatment is required. Especially chronic or long-term patients with those diseases are considered co-disease and are treated for that. (Curcio, Tria et al. 2016, Diuk-Wasser, Vannier et al. 2016). Co-infections

between *B. duncani* and BBSL have also been suspected and may as well be a common (Horowitz and Freeman 2019, Parveen and Bhanot 2019)

The definition of a co-infection should certainly include two significant pathogens causing disease but can in some situations not be completely easy to differ from immunizations and post-infections were seropositivity has been found. This can be the case in *B.* spp. infections. However, co-infections have in one review been described in clinical situations of high fever and erythema migrans (EM) and patients with neurological symptoms connected to neuroborreliosis and TBE (Boyer, Lenormand et al. 2022).

Aims of the present investigation

The overall aims of the study were to investigate the presence of the tick-borne pathogens *Babesia* and TBEV in the Southern part of Sweden. The background to this were reports of presence of *Babesia* species in ticks in Sweden and sporadic cases of human babesiosis described in Sweden as well as reports of significant numbers of seropositive individuals in other parts of Europe and many clinical cases of human babesiosis in the US. Also, reports of TBEV have increased in new areas, including Southern Sweden.

Many different techniques have been used during this thesis work, but primarily different antibody assays were used. In vitro studies of *Babesia* parasites in experimental cell cultures and with the possibility of applying it into antibody assays have also be performed.

In conclusion the specific aims of the three studies included in this thesis were:

1. Perform an epidemiological study to investigate how common *Babesia* antibodies are in humans in Southern part of Sweden. Compare different populations such as tick-bitten individuals with a healthy control group.
2. Perform an epidemiological study to investigate how common TBEV antibodies are in humans in Southern part of Sweden. Compare different populations such as tick-bitten individuals with a healthy control group.
3. Create a manageable and reproducible ELISA for *B. divergens*, which can be used both in routine clinical practice and for research purposes. To establish a method to purify *Babesia* merozoite antigen extract and use this extract to set up an ELISA method and use this for a small study of the seroprevalence of *B. divergens* antibodies in Southern Sweden.
4. Investigate in vitro preferences of *Babesia* parasites into human RBC.

Materials

Study sampling

Different sampling projects were performed in the studies to form the different cohorts used in the studies. Some sample cohorts were already existing, while others were actively designed and collected specifically for this project, such as the sampling of anonymous individuals from rural areas in Skåne (AIRA) and tick-bitten individuals (TBI) groups.

Paper I and III: Sampling from previously *Borrelia burgdorferi* infected persons and a control group

In cooperation with the Biological Specimen Bank of the Department of Clinical Microbiology, Skåne, samples from previously *Borrelia burgdorferi sensu lato* (BBSL) infected persons were collected.

In total, 100 individuals were informed by letter and were offered to decline participation, however, none of 100 disagreed to be included in the study. The patients were from primary health care centers and also hospital inpatients. The participants came from all over Skåne. In comparison with this cohort, a control group (CG) of 197 samples was collected at Clinical Chemistry in Region Skåne. These samples were from volunteers such as employees and students, aged 18-67, and we did not know anything about previous TBD. All participants stated good physical health for the moment and no treatment with regular medications. All samples were stored at -80°C.

Paper II: Sampling project from anonymous individuals (AIRA) and tick-bitten individuals (TBI)

A major collection of samples was initiated during 2016-2017. The aim was to collect and organize a well-prepared study material from adequate individuals to use during the PhD project. It was organized together with Clinical Chemistry, and before that an ethical permission was obtained from the Regional Ethical Board in Lund.

The collection included two parts. Part one included anonymous samples from health care centers (Sw. vårdcentraler) in rural areas of Skåne (AIRA). The selection was made in the hospital laboratories of Kristianstad and Hässleholm based on site of sampling, and neither age nor gender were considered. All personal data was eliminated by the laboratory staff before inclusion in the study. The health care centers were situated in northern and eastern parts of Skåne: Tyringe, Knislinge, Broby, Bromölla, and Osby. These are relatively rural areas where some of the areas have had most of the clinical TBE cases in Skåne (Knislinge, Broby and Bromölla).

Part two consisted of volunteer participants who according to themselves "often received tick-bites". They were recruited through public information. It was published on social media homepages (Facebook etc.) of Skåne Regional Council and many volunteers were recruited through advertising through the regional branch of the Swedish orienteering association. Important information about the study participants were included (age, gender, residence, previous TBEV-vaccination) and different study material such as blood smears, serum and plasma were taken (table 3). This sampling was conducted by experienced biomedical technicians, trained in sampling, preparing, and storing biochemical samples. Cooperation with all ten hospital laboratories in Skåne was established for sampling and organization of the material.

Table 3. Overview of cohorts included in the thesis

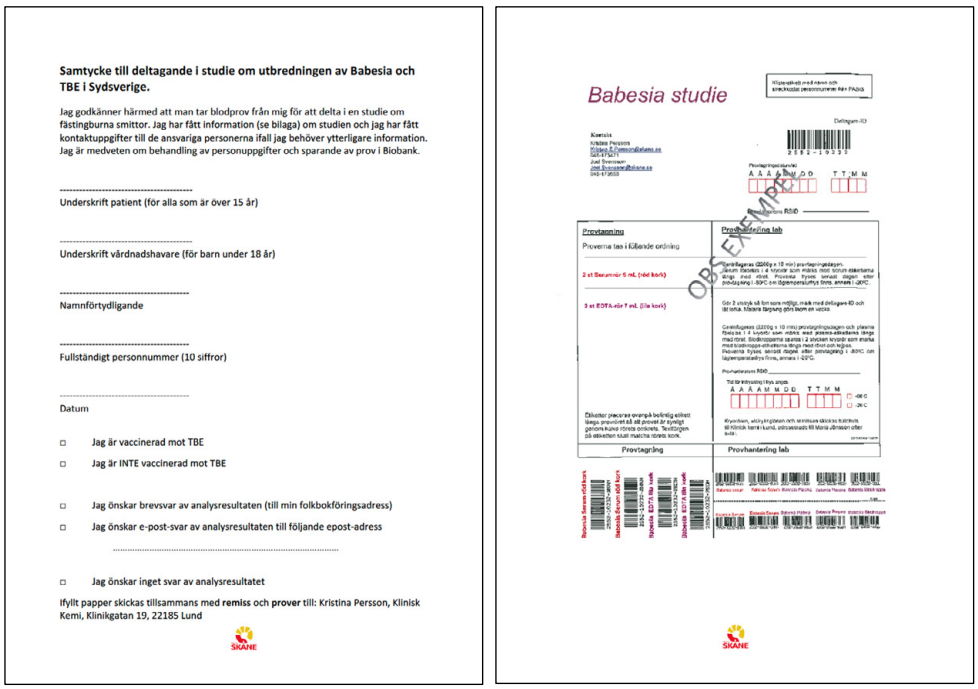
Cohort:	n:	ID:	Storage and material:	Collected:	Paper:
Borrelia 2015 (BBAP)	100	1-106	-80°, serum	2015	I, III
Control group (CG)	189	1-40, 1001-1061, 2003-2100	-80°, plasma, serum,	2014-2015	I, III
Tick-bitten individuals (TBI)	198	9 digits ID: 2552XXXXX / 9999XXXXX	-80°, separated blood cells, blood smear	2016-2017	II
Anonymous individuals from rural areas (AIRA)	491	1001X-1542X	-80°, separated blood cells, blood smear	2016-2017	II

Ethical permissions

The withdrawal of samples from the Biological Specimen Bank of Clinical Microbiology was ethically approved by the Regional Ethical Board in Lund, Sweden (reference 2014/659) and by the Regional Board for Quality Register (S-KVB). The samples were handled anonymously with no traceability.

The sampling project from health care centers and tick-bitten individuals was ethically approved by the Regional Ethical Board in Lund, Sweden (reference

2017/177) and by the Regional Board for Quality Register (S-KVB). The samples were given an anonymous study identification number after inclusion in the study. In cases where the study participants had demanded feedback for positive laboratory results, a code key could be used to trace data connected to those individuals. The key code was kept locked up. In these cases, this was accomplished according to the participant agreement from the beginning (figure 6).



Samtycke till deltagande i studie om utbredningen av Babesia och TBE i Sydsverige.

Jag godkänner härmed att man tar blodprov från mig för att delta i en studie om fästingburna smittor. Jag har fått information (se bilaga) om studien och jag har fått kontaktuppgifter till de ansvariga personerna ifall jag behöver ytterligare information. Jag är medveten om behandling av personuppgifter och sparande av prov i Biobank.

.....
Underskrift patient (för alla som är över 15 år)

.....
Underskrift vårdnadshavare (för barn under 18 år)

.....
Namnförtydligande

.....
Fullständigt personnummer (10 siffror)

.....
Datum

- ☐ Jag är vaccinerad mot TBE
- ☐ Jag är INTE vaccinerad mot TBE
- ☐ Jag önskar brevsvar av analysresultaten (till min folkbokföringsadress)
- ☐ Jag önskar e-post-svar av analysresultaten till följande epost-adress

.....

- ☐ Jag önskar inget svar av analysresultatet

Ifyllt papper skickas tillsammans med **remiss** och **prover** till: Kristina Persson, Klinisk Kemi, Klinikgatan 19, 22185 Lund

SKÅNE

Babesia studie

Provtagning

Provtagning lab

OBS EXEMPEL

Provtagning

Provhantering lab

SKÅNE

Figure 6: Participant formula and referral formula.

Methods

Paper I: Laboratory procedures

An assay for antibodies against BBSL was used for both IgG and IgM. Positivity was determined as either an elevated IgG concentration (≥30 AU/mL, chemiluminescent immunoassay, LIAISON® *Borrelia burgdorferi*, DiaSorin) or an elevated IgM index (> 1.1). When an elevated IgM index was seen, an immunoblot (EUROLINE-WB Euroimmun AG) was also performed and confirmed it as *Borrelia* positive. This two-tier protocol is in accordance with the current standard recommendations (Dessau, van Dam et al. 2018). The samples were assessed in the routine laboratory of Clinical Microbiology in Skåne and were not reassessed in this study.

Assays for *B. divergens* and *B. microti* were performed for this study using indirect fluorescent antibody (IFA) tests. The assay for *B. divergens* was an internal assay previously used by a co-author (Hunfeld, Lambert et al. 2002) and developed in another study (Kampen, Maltezos et al. 2002). Firstly, isolates from *B. divergens* infected cattle were infested in Gerbils (Meriones unguiculatus) and antigen was harvested at a parasitemia of 20%. After washing in PBS, the diluted iRBC were transferred onto glass slides and used without further fixation. The IFA was done with FITC-labelled anti-human IgG immunoglobulin (MRL Diagnostics). A commercial test was used for *B. microti* (*B. microti* IFA IgG/IgM), MRL Diagnostics®, Cypress, California). The kit includes golden hamster (Mesocricetus auratus) erythrocytes that have been infected with a strain that was originally isolated from a human patient. For both assays, sera were titrated on different days (in triplicates) and the results were the mean of the three experiments. Positive and negative controls were used in both assays. The cut-off values for IgG directed against *B. divergens* and *B. microti* were ≥1:128 and ≥1:64, respectively. The specificities for anti-*B. microti* and anti-*B. divergens* IgG detection were 98.6% and ≥97.5%, respectively. If the IgG result was >1:32, IgM was also performed (Hunfeld, Lambert et al. 2002).

Paper II: Laboratory procedures

The serum samples were analyzed for TBEV IgG and IgM at Clinical Microbiology, Region Skåne, during two separate days in 2018 in the routine laboratory. The assay is an enzyme-linked immunosorbent assay (ELISA) from Euroimmun AG, Germany. The cutoff for a positive test was set to ≥20 RU/mL for IgG, and for IgM an index of ≥1.1 was considered positive. 0.8 to <1.1 was regarded as borderline positive and <0.8 was negative. Samples from tick-bitten individuals (TBI) and samples from anonymous individuals (AIRA) with positive IgM-index and/or positive IgG-level were passed on for a TBEV neutralization test (NT). This test was done by the Public Health Agency of Sweden, according to (Vene, Haglund et al. 1998) and a value of ≥5 was regarded as positive. A positive response in this test indicates that the patient had a TBEV infection. The test does not respond to vaccinations.

Paper III: Laboratory procedures

Bovine *B. divergens* isolate and *in vitro* culture in human RBC and merozoite extraction, ELISA, and Western Blot

Bovine *B.* divergens isolate was cultured in vitro in human RBC. After establishment in culture medium merozoite extraction was performed. Samples were later sent to University of Zurich, Switzerland, where PCR and sequencing confirmed the parasites to be *B. divergens* (Teal, Habura et al. 2012). ELISA assays were performed by a multi-step assay described in paper III. Western blots were performed by merozoite extract proteins were separated on SDS-PAGE gels followed by further analysis.

Other methods used

Indirect Fluorescence Antibody assay (unpublished)

A commercially available assay for indirect fluorescence antibody assay (IFA) has been performed on some of the samples (figure 7). The assay manufactured by Fuller Laboratories ®, CA, US, catalogue number BmG-120 has been used before in previous evaluations (Chisholm, Ruebush et al. 1978, Krause, Telford et al. 1994, Krause, Ryan et al. 1996, Ryan, Krause et al. 2001, Lempereur, Shiels et al. 2015,

Pokhil, Bondarenko et al. 2020). Assays both for IgG and IgM were tried out but there was some uncertainty around conclusions for specificity and the coating between the slides. Therefore, we concluded that we needed to use a better method like for example an ELISA that is more reproducible, to be able to evaluate antibodies against *Babesia.*

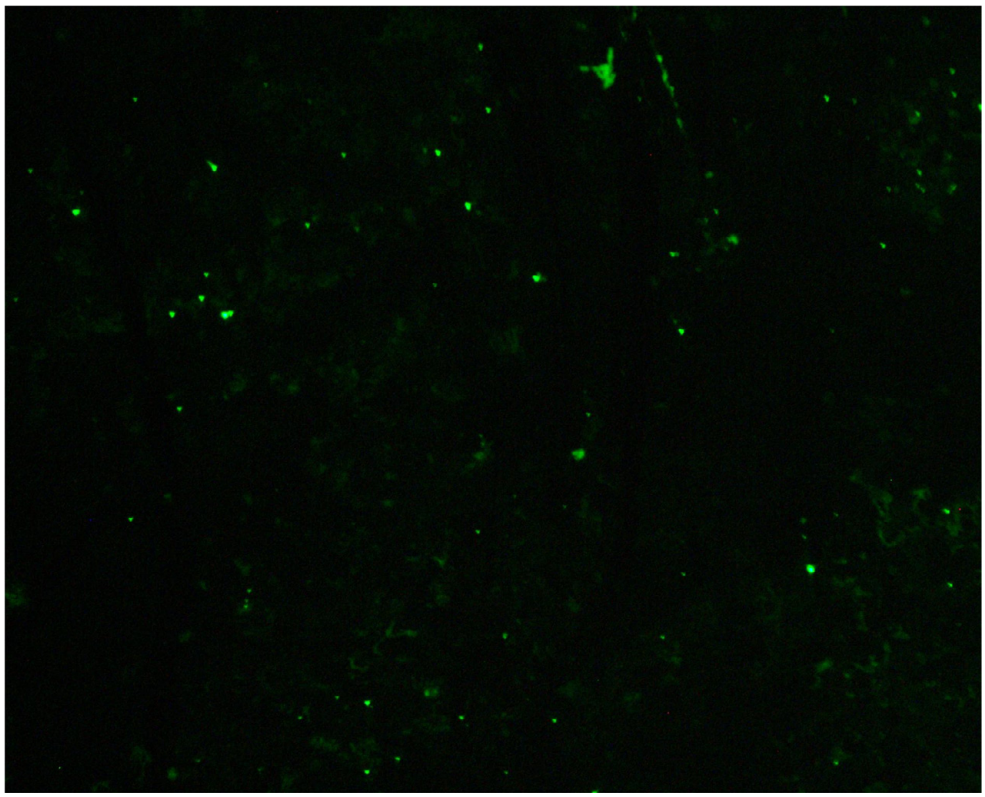


Figure 7: IFA. Photo J. Svensson

PCR (unpublished)

Polymerase chain reaction (PCR) was tested during the project (figure 8). A commercial kit from Immundiagnostik AG, MutaGEL® was used, which has the intended use for detecting *B. divergens, B. microti* and *B. odocoilei*. The assay was estimated as more usable and easy to handle than more complex assays described (Chan, Marras et al. 2013, Wang, Villafuerte et al. 2015, Wang, Wormser et al. 2015, Akoolo, Schlachter et al. 2017, Rozej-Bielicka, Masny et al. 2017, Stanley, Stramer et al. 2021). A single amplification product of 210 base pair of length was

detected in an agarose gel. The assay was performed on material from a known case previous described (Bläckberg, Lazarevic et al. 2018).

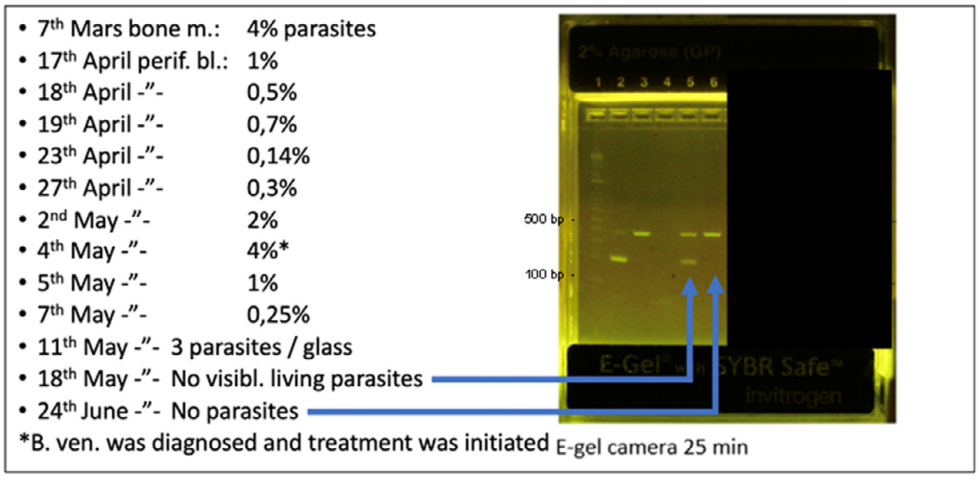


Figure 8: PCR assay for *B. venatorum* compared with percentages from blood smear examinations. Lane 1, 10: ladder with molecular weight. Internal control band at 400 bp and *B.* spp. band at 210 bp. Lane 2: pos control, Lane 3: neg control, Lane 4: blank sample, Lane 5: positive sample in the middle of treatment regime. Lane 6: convalescent sample. Source: J Svensson (previous unpublished results)

Microscopy (unpublished)

The traditionally most used method to detect and categorize intraerythrocytic parasites is with thin blood smears using microscopy. The normal procedure is with Giemsa-stained smears. However new techniques have been developed in recent years to digitalize blood smears and in connection with this also attempts to be able to perform automatized and digitalized identification of intraerythrocytic parasites. The method still seems to be insufficient and requires conventional microscopy for accurate detection (Yoon, Kwon et al. 2019). Our attempts (not yet published) were an examination of material from paper III were blood smears from the AIRA and TBI groups were investigated after digital flags had alerted for manual review, but no further *Babesia* detection could be confirmed.

Statistics

In paper I, the chi-squared statistical test was used to compare the results of the seropositive individuals in the BBSL positive group and the control group. In paper II, p-values were calculated with two-samples t-test when comparing different

means of IgM and IgG between geographically and age-divided populations. P-levels of <0.05 was considered as statistically significant. Analyses were performed with SPSS version 19.0 software (SPSS Inc., Chicago, IL).

In paper II, p-values were calculated with two-samples t-test when comparing different means of IgM and IgG between geographically and age-divided populations. When comparing the results of the seropositive cohorts in percentages the chi-squared statistical test was used. A p-value of <0.05 was considered as statistically significant. Analyses were performed with SPSS version 19.0 software. (SPSS, Inc., Chicago, IL).

ELISA assays (paper III) were monitored by following standard deviations (SD) and coefficient of variation (CV) (percentage) for positive and negative controls between different runs. Hence trends of analysis imprecision were monitored. Calculations were performed in the software Microsoft Excel ®.

Results

Paper I

Two cohorts were compared: BBAP and the healthy control group (CG). The mean ages of the participants were 57 and 40 years, respectively, and the age ranges were 5–87 and 18–66 years. In the BBAP group there was a majority of men (58%), while among the healthy controls there were slightly more women (62%). In the BBAP group, 65 of the 86 samples were obtained from patients who visited primary health care centers; and 21 samples were from hospital inpatients.

From the 86 samples in the BBAP group, 73 and 13 were seropositive for IgG and IgM against BBSL, respectively. Among these patients, the number of *Babesia* seropositive (IgG) individuals was 11 and 3, respectively. In total the seroprevalence was 16.3% for *Babesia* antibodies among the BBAP patients. Twelve of the 65 samples obtained from primary healthcare centers were positive for *Babesia* antibodies, as were 2 of the 21 inpatient samples which were from infectious diseases and neurological clinics.

Table 4: Summative comparison between BBAP and CG.

	Total number of individuals with *Babesia* IgG (*B. divergens* and *microti*)	**Percentage *Babesia* IgG positive individuals**
Borrelia positive group (BBAP), n=86	14	16.3%
Healthy controls (CG), n=197	5	2.5%

Chi-square-test with p-value of 0.000022 between the groups.

From the 14 samples that were positive for *Babesia* antibodies in the BBAP group, 5 and 8 were positive for *B. divergens* IgG and *B. microti* IgG, respectively. Further, one individual had a positive reaction in both assays, and 1 patient also had a positive *B. microti* IgM titer. For all individuals in the BBAP group, the positive titers for *B. divergens* were 1:128; while the positive titers for *B. microti* showed a larger variation with one titer reaching 1:512. From the 14 samples that were positive for *Babesia* antibodies in the BBAP group, 6 were from females (3 positive for *B. divergens* and 3 positives for *B. microti*).

The geographical distribution of the *Babesia* antibody positive individuals was relatively evenly distributed throughout the Skåne County (figure 9).

In the healthy control group, 5 of the 197 individuals were seropositive for *Babesia* antibodies. Two and 3 were positive for *B. divergens* and *B. microti,* respectively. The total seroprevalence for *Babesia* antibodies among the healthy individuals was 2.5%. All 5 seropositive individuals in the group were female. Comparison of the BBAP group with the healthy volunteers revealed that 16.3% and 2.5%, respectively, of the individuals had a positive titer for at least one of the *B.* spp. This difference is statistically significant with a p-value of 0.000022 (table 4).

In conclusion, the results show high seroprevalence of *B.* spp. among BBAP with a fairly even distribution between *B. divergens* and *B. microti* and also some seropositive individuals among the healthy controls.

Paper II

Two cohorts were compared: Tick-bitten individuals, TBI and anonymous individuals from rural areas, AIRA, collected from from five different primary health care centers in Northern Skåne County. The cohorts consisted of 198 and 491 participants, respectively.

In the TBI group the overall TBE vaccination coverage was 45% according to the study questionnaires. Ninety-two individuals were vaccinated, 99 were unvaccinated, and 9 had no or unclear response to prior completed vaccination. When the IgM-indices were measured, two samples were positive for TBEV (1%) and three samples had borderline results (1.5%). The overall mean and median for the IgM-indices were 0.12 and 0.07, respectively, and due to the low values, no further comparisons were made. For IgG, 57 individuals (28%) were positive. The overall mean and median for IgG were 28.2 and 4.0 RU/mL, respectively. IgG means for vaccinated and unvaccinated were 55.5 and 3.7 RU/mL, respectively (p value <0.001) (Fig. 2). The TBI group was further divided into three age cohorts with IgG means of 37.3 RU/mL in the youngest group (<50 years, n = 68), while the middle-aged group (50–69 years, n = 86) had a considerably lower mean level of IgG, 19.2 RU/mL (p = 0.018 compared with the group <50 years). The oldest individuals (>69 years, n = 44) had an IgG-level of 34.2 RU/mL. There was no statistically significant difference in vaccination coverage between the age groups: 44% in the youngest, 44% in the middle-aged, and 55% in the oldest, respectively.

Within the TBI group, there was a similar distribution between men and women and no significant differences according to sex for means of IgM indices or IgG levels (IgG means was 58.5 RU/mL for men and 54.5 RU/mL for women). Forty-four percent of females and 57% of males were vaccinated and had completed the

vaccination program. The IgG mean value for the TBI group was significantly higher compared to the AIRA group (p value <0.001).

In the AIRA group it was as follows: When analyzing the IgM-index, three samples were positive for TBEV (0.6%) and three samples had borderline results (0.6%). For IgG, 27 individuals (5.4%) were positive. The overall mean and median for the IgM-indices were 0.09 and 0.05. Since the IgM indices in general were very low, no further comparisons were made. The overall IgG seropositivity was 5.3%, ranging from 0% to 8.6% in different geographical locations (Fig. 1). There were no significant differences between the locations. The overall mean and median for IgG was 8.6 and 2.0 RU/mL, respectively. The mean values for the different locations were compared and differences were found when Bromölla (IgG= 10.7 RU/mL, n = 152), Knislinge (IgG= 12.7 RU/mL, n = 105), and Tyringe (IgG= 6.1 RU/mL, n = 142), which all had higher values, were compared with the lower mean value in Osby (IgG= 2.1 RU/mL, n = 44). Broby had a mean value in between of IgG 6.5 RU/mL (n = 48). Statistically significant differences between the IgG means were also found between Bromölla and Osby (p = 0.001), between Knislinge and Osby (p = 0.006), and between Tyringe and Osby (p = 0.002) (figure 9).

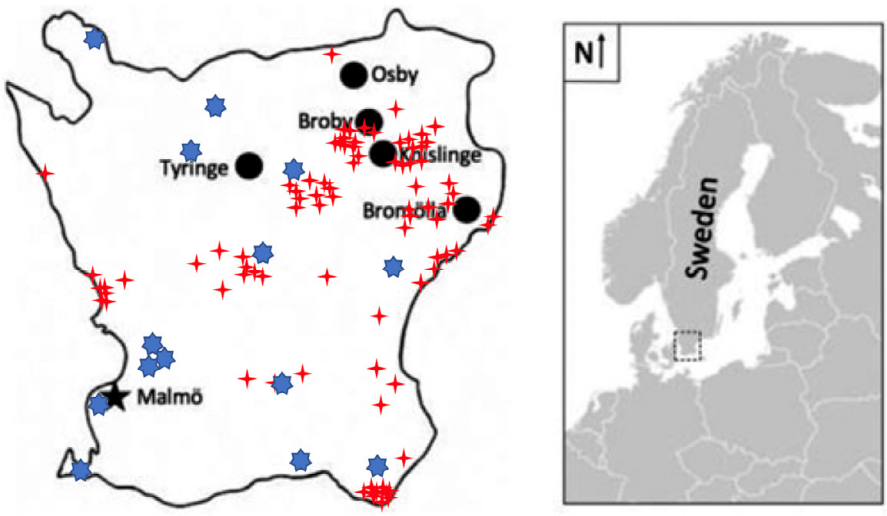


Figure 9: Combined map: Black dots: Our five sampling locations for the AIRA samples. Probably most participants lived in that community or in the surroundings (study 2) Red crosses: registered clinical cases of TBE 2000-2021 (study 2). Blue stars: Seropositive Babesia samples, notice that in this case it is the location of the refering clinic of the sample where Borrelia burgforferi was seropositive. (study 1) (reference: 1177's e-services based on statistcs from FoHM, 2023-03-02). Map: J. Svensson.

In the TBI group, six individuals had positive or borderline values for IgM or IgG, despite that they had reported not to have been vaccinated. Samples from these individuals were analyzed using a TBEV Neutralization test (NT), and we found two individuals who had positive results (which they were informed about according to the study protocol). In the AIRA group, the vaccination status was unknown, but the samples that were positive or borderline for IgM were analyzed using the NT. One of the individuals tested in NT was found to have a clearly elevated titer of >160.

In conclusion, the results show among anonymous samples a seroprevalence of 5.3%, but in a group who often among tick-bitten individuals a seroprevalence of 28% (45% self-reported as vaccinated).

Paper III

When slides were inspected 24 hours after addition of human RBC to the cow RBC*,* *Babesia* parasites had started to invade the human RBC. They could be seen as trophozoites, paired shapes, and double trophozoites. After about three weeks a healthy parasite culture in human RBCs was seen. Thereafter periods of crisis occurred in which parasitemia was consistently low and fewer developmental stages were observed. The culture became healthy afterwards where it was possible to achieve 40% parasitemia or more. Purification of merozoites could thereafter be performed for later application in ELISA and Western blot assays. In the ELISA, higher seroprevalences could be seen in the *Borrelia* positive group compared with a control cohort. We tested a few of the positive samples in Western blot and could see that there were clear reactions against bands when using merozoite extract, indicating that the positive response in the ELISA is due to antibodies directed against merozoite antigens and not against RBC antigens. There was a very good correlation between the titer in the commercial IFA kit and our ELISA results assay for *B. divergens,* indicating that our assay can be used instead of IFA for measurement of antibodies against *B. divergens,* which would facilitate evaluation of exposure to this parasite. However, further studies including more controls are needed to be able to evaluate this assay more thoroughly.

Discussion

Babesia

Strengths and limitations of the studies

In our paper I the surprisingly high percentage of antibodies for *B. divergens* and *B. microti* among *Borrelia* antibody positive individuals include Southern Sweden as an area with likely presence of *Babesia* species among ticks and other reservoirs. The increasing number of ticks has already been showed in this area as well as significant percentages of *B.* spp. infested in the ticks (Jaenson, Jaenson et al. 2012, Karlsson and Andersson 2016). The used IFA assay has been used in several other studies (Hunfeld, Lambert et al. 2002, Rigaud, Jaulhac et al. 2016). Cross-reactions are well explored in general and there are no clear signs of that occurring in this assay, even though some elements of cross-reactions related to autoimmune or infectious conditions can occur in specific cases as for all antibody based diagnostic assays. Similar controls have been performed with the ELISA assay (paper III) and the combination of tests can further strengthen the knowledge on an epidemiological level about whether *Babesia* is present in an area or not.

From a clinical perspective, it is more uncertain what the results implicate. Many of the immunized individuals probably had a self-limiting course of the infection. Factors that help healing the infection are to have intact splenic function, intact immunocompetent functions with leukocytes and immunoglobulins and to not be elderly. However, the healthcare system meets and treats a lot of individuals with unspecific symptoms such as fever and chills, myalgia and also renal impairment and haemolysis, and sometimes these symptoms might be chronic or periodic. Some of these symptoms might be due to *Babesia*, while others might be due to other unknown pathogens. It can also be supposed that some of the standard treatments for borreliosis may have some but far from ideal effects on human babesiosis which could potentially limit some of the sickest cases to develop into a more severe disease.

Today, in probably most cases of human babesiosis the diagnosis is not considered in Sweden because both diagnostic tools and knowledge are absent. Since we now can conclude that *B. microti* is established here and the situation in North America shows the symbiotic co-existence between *Borrelia burgdorferi* and *B.* spp., and the

reported numbers of human borreliosis and babesiosis are about 10 to 1, both diagnoses should be considered in relevant cases also in Sweden.

In paper III the parasite used for the ELISA was confirmed both by microscopy in our laboratory, and by an external routine laboratory that showed PCR confirmation of *B. divergens.* This strengthens the specificity of the developed ELISA. Further we tried to evaluate potential conditions that could cause cross-reactive antibodies such as other infectious diseases, and samples from malaria endemic areas. Of course, there is always the possibility of testing more potentially cross-reactive conditions to further strengthen the assay. Other concerns are about the cell culture procedure which can be performed in various manners and different protocols have been suggested. For instance, culture medium content, gas composition and additions of antimicrobic substances can be different (Erp, Smith et al. 1980, James, Levy et al. 1981, Grande, Precigout et al. 1997, Schuster 2002, Malandrin, L'Hostis et al. 2004), but after many trials using different conditions we found a protocol that worked well to relatively easily get access to pure merozoites, without using human serum or getting too much haemoglobin or red cells in the purified extract.

Significance of the studies

Before, high percentages of seroprevalence have been seen for anti-*Babesia* antibodies in different countries in Europe. With the present study (paper I), this has now also been shown in Southern Sweden. In other parts of Europe such as Poland, Germany, Belgium, and Italy investigations of cohorts with risk factors for tick bites for example forestry workers, have been done to search for tick-borne diseases. Primarily *B. divergens* have been discovered but more lately also *B. microti* which is also strengthened by our present study (paper I).

Only sporadic clinical cases have been reported in Europe even though many of them have illustrated very sick patients and lethal cases. For immunocompetent individuals the question arises about the degree of pathogenicity from the different *Babesia* species. And even if the pathogenicity is significant and considerable the lack of knowledge and reported cases may depend on several factors as follows:

- Lack of awareness from the conventional healthcare system including absence of education in the field in medical programs and specialist training programs.
- Lack of awareness in general population. The public experience of tick-borne diseases is in many countries and regions for instance concentrated to *Borrelia burgdorferi*, TBEV and some others.
- Requirement of reporting the disease to authorities. Many other infectious diseases have mandatory reporting systems to public authorities, but this is not the case when it comes to human babesiosis in most countries in Europe.

Comparing to i.e., the US the disease is much more well followed and monitored by the authorities (CDC).

- Human babesiosis may be easily overlooked and may not be recognized in immunocompetent patients due to the non-specific symptoms and due to the generally low awareness in the health care system.
- *B. microti* and *B. divergens* should be considered with parallel approaches since the different species don't show cross reactive patterns.
- There is a potential risk for transfusion transmitted disease of *Babesia* species in northern Europe since no screening routine is established.
- The ability to find low parasitemia's would be facilitated by using PCR, especially for screening for *Babesia* species in transfusion medicine as well as in clinical cases, especially when parasitemia is fluctuating as has been seen in clinical cases (Bläckberg, Lazarevic et al. 2018).

TBEV

In paper II the presence of TBEV antibodies was examined in different cohorts. High percentages of IgG levels were seen in areas which have previously been considered as risk areas as well in other areas. This contrasted with other areas where very small percentages were seen. This is line with the pattern of TBEV establishment in hot-spots or limited geographical areas (Agergaard, Rosenstierne et al. 2019, Waldeck 2019, Liebig, Boelke et al. 2021).

The levels of immunoglobulins are as well of interest. The middle-aged group of 50–69 years had lower levels of IgG than younger and older groups. The explanation may be by a lower exposure to TBEV from tick-bites. Another reason may be lower immunoglobulin levels. A study have shown that older people (>60 years) develop lower levels of IgG (Lock and Unsworth 2003). Other factors that may influence is that in Sweden it is plausible that older people (retirement age is about 65 years) are spending more time in the nature and forests where there are ticks. This combined with a weaker immune defense could make them more vulnerable to be infected. The study itself also shows a higher vaccination frequency (55% compared with 44% in the other two groups). The study also revealed a clinical case of a patient with long-term problems who had experienced extensive examinations for neurological symptoms, which now was explained with findings of a positive TBEV test.

Conclusions

The main conclusions from this thesis are listed below.

- Our results show a significant and surprisingly high percentage of individuals with *Babesia* antibodies among individuals in Southern Sweden seropositive for *Borrelia burgdorferi*: 16.3% in this group compared to 2.5% in a healthy control group.
- What also is remarkable is that several of the individuals with *Babesia* antibodies had antibodies against *Babesia microti,* which has mostly been highlighted in North America before. These findings reveal that there is an increasing need to broaden awareness of the prevalence of different *Babesia* species in northern Europe.
- There should be awareness of *Babesia* when a patient has unclear symptoms after blood transfusions.
- Considerable differences in TBEV IgG between different groups of people were shown. High IgG levels were found in both risk areas and in other areas, which not only can be explained by different vaccination coverages.
- Our results motivate an increased awareness of TBE in Southern Sweden and that TBEV vaccination should be considered for people who are often outdoors in risk areas.
- In conclusion, both babesiosis and TBE are highly relevant to consider in the clinical context!

Future perspectives

- Investigate in vitro the invasion preferences of *Babesia* parasites into human RBC of many different blood groups, to be able to understand the invasion process of the parasite and thereby find new medications against *Babesia*.
- Perform a long-term study of patients with *Babesia* and investigate antibody-dependent immunity against the different *Babesias in vitro*.
- Investigate if there are differences in blood groups in individuals that have had *Babesia*, compared to those that did not.
- Cooperate with the health care system and the public society with information about the pathogen and disease.
- Cooperate with the health care system to offer improved diagnostic opportunities for the most common *Babesia* species such as *B. divergens, B. venatorum* and *B. microti.* This should primarily include serological diagnostic opportunities and direct detection such as PCR.
- Cooperate with authorities for investigating the possibility of requirement of mandatory reporting for clinical confirmed cases of human babesiosis in Sweden to FoHM (Folkhälsomyndigheten, The Public Health Agency of Sweden).

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