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Establishment of *Babesia* laboratory model and its experimental application

Ph.D. thesis

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Jalovecka, M. 2017: Establishment of *Babesia* laboratory model and its experimental application. Ph.D. Thesis, in English - 180 pp., Faculty of Science, University of South Bohemia, České Budějovice, Czech Republic.

Annotation: Growing incidence of infections caused by the tick-transmitted protozoan parasite *Babesia* spp. defines babesiosis as an emerging disease from the aspect of human and veterinary medicine. The thesis provides an insight to biology of two main agents of human babesiosis, *Babesia microti* and *Babesia divergens*. We introduce here the fully optimized quantification model of *Babesia* parasite enabling the detailed investigation of the parasite developmental cycle and identification of molecules playing a role in its acquisition and transmission by the vector *Ixodes ricinus*. Novel and detailed information about *Babesia* dissemination within the tick tissues are given by newly implemented visualization and quantification techniques. Special emphasis is paid to parasite development in the tick salivary glands, the primary site responsible for parasite transmission from the vector into the host. Using gene-specific silencing we screene the tick immune pathways including effector molecules and evaluate their role in *Babesia* acquisition. We also provide a detailed view to *Babesia* parasite sexual commitment by monitoring its kinetics upon various stimuli. Moreover, a new direction of anti-babesial therapy is proposed by validation of the *Babesia* proteasome as a drug target. Overall, the research presented in the thesis extends the current knowledge of the *Babesia* parasite biology including molecular interactions at the tick-*Babesia* interface and thereby could significantly contribute to a potential control of babesiosis.

Jalovecka, M. 2017: Establishment of *Babesia* laboratory model and its experimental application. Ph.D. Thesis, in English - 180 pp., Doctoral School of Biology and Health, University of Bretagne-Loire, ONIRIS, Nantes-Atlantic College of Veterinary Medicine and Food Sciences and Engineering, Nantes, France.

Annotation: Du fait de l'incidence croissante des infections à *Babesia* spp., Protozoaire parasite transmis par les tiques, la babésiose est considérée comme maladie émergente en médecine humaine et vétérinaire. Cette thèse apporte un aperçu de la biologie de deux agents principaux de la babésiose humaine, *Babesia microti* et *Babesia divergens*. Un modèle quantifié du parasite *Babesia* est proposé, permettant une étude détaillée de son cycle de vie et l'identification de molécules jouant un rôle dans son acquisition/transmission par le vecteur *Ixodes ricinus*. Des informations nouvelles et détaillées sur la dissémination de *Babesia* dans les tissus de la tique sont apportées par les techniques de visualisation/quantification développées. Le développement parasitaire dans les glandes salivaires est particulièrement étudié, car premier site responsable de la transmission du vecteur à l'hôte. Par inactivation ciblée de gènes, nous avons exploré les voies du système immunitaire de la tique, dont les molécules effectrices, et évalué leur rôle dans l'acquisition de *Babesia*. Une étude détaillée de l'engagement de *Babesia* dans la phase sexuée est réalisée, en suivant sa cinétique sous l'effet de divers stimuli. De plus, une voie nouvelle de lutte antiparasitaire est proposée en validant le protéasome de *Babesia* en tant que cible thérapeutique. D'une façon générale, les travaux présentés élargissent les connaissances actuelles de la biologie du parasite *Babesia* incluant les interactions moléculaires à l'interface tique-*Babesia* et contribuent donc au contrôle de la babésiose.

Declarations

I hereby declare that this thesis is based on my own work and all other sources of information have been acknowledged. I hereby declare that, in accordance with Article 47b of Act No. 111/1998 in the valid wording, I agree with the publication of my PhD thesis, in full to be kept in the Faculty of Science archive, and in shortened form resulting from deletion of indicated parts in electronic form in publicly accessible part of the STAG database operated by the University of South Bohemia in České Budějovice accessible through its web pages. Further, I agree to the electronic publication of the comments of my supervisor and thesis opponents and the record of the proceedings and results of the thesis defence in accordance with aforementioned Act No. 111/1998.
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List of publications and author's contributions

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MJ participated on co-writing the publication.

Aase A, Hajdusek O, Øines Ø, Quarsten H, Wilhelmsson P, Herstad TK, Kjelland V, Sima R, Jalovecka M, Lindgren PE, Aaberge IS. Validate or falsify: Lessons learned from a microscopy method claimed to be useful for detecting *Borrelia* and *Babesia* organisms in human blood. Infect Dis. 2016;48(6):411-9. (IF = 1.119)

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MJ designed and conducted the experiments, wrote the manuscript and acted as a corresponding author.

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MJ conducted the experiments and wrote the manuscript.

Jalovecka M, Hajdusek O, Kopacek P, Malandrin L. Life cycle of piroplasms: comprehensive analysis. *In preparation*.

MJ wrote the manuscript and will act as a corresponding author.

The thesis contains unpublished data which are thoroughly described and discussed. The obtained data are intended to be published once fully completed.

MJ participated in a design and performance of the experiments and will write the manuscript(s).

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PART I. General introduction

Part I. provides a comprehensive review on current knowledge of the problematics.

In this part, two review publications are included, one published [1] and one in preparation:

Hajdusek O, Sima R, Ayllon N, Jalovecka M, Perner J, de la Fuente J, et al. Interaction of the tick immune system with transmitted pathogens. Front Cell Infect Microbiol. 2013;3:26. doi: 10.3389/fcimb.2013.00026. PubMed PMID: 23875177; PubMed Central PMCID: PMCPMC3712896.

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Piroplasms, the tick-transmitted apicomplexan parasites

The group *Piroplasmida* refers to intracellular tick-transmitted parasites which received its name after the pear-shaped (piriform) intra-erythrocytic stages [2]. Piroplasms possess a great economic, veterinary and medical impact as one of the most common blood parasites of mammals. The disease caused by piroplasms is considered as emerging due to the growing incidence in humans, livestock, wildlife, and pet animals [3-8].

The taxonomic classification places *Piroplasmida* species in the phylum *Apicomplexa*. Based on multi-gene analyses, three genera – *Babesia*, *Theileria* and *Cytauxzoon* – form the order *Piroplasmida*, closely related to *Plasmodium*, an agent of malaria disease (e.g. [9-12]). Shared morphological and developmental hallmarks of *Piroplasmida* are apical complex organelles, a merogony (asexual multiplication) within erythrocytes of vertebrate hosts and sexual multiplication followed by sporozoites formation in the invertebrate hosts [13]. Classical taxonomy assigned the *Babesia* as a sister clade of *Theileria* (and *Cytauxzoon*) based on morphological and/or developmental features (e.g. [14, 15]). Widely used criteria referred *Babesia* species as organisms transmitted via tick larval progeny (transovarial transmission) and multiplication restricted to host erythrocytes [16]. Alternatively, *Theileria* and *Cytauxzoon* are limited to only transstadial transmission in vector ticks and undergo the schizogony in nucleated blood cells prior the infection of erythrocytes [5, 12, 17]. Nevertheless, such classification is limited and does not reflect the evolution and diversity of *Piroplasmida* species as become apparent after molecular data availability (e. g. [5, 12, 15, 18-20]). Based on *18S* gene sequence, the *Piroplasmida* phylogeny was thoroughly characterized (e.g. [5, 18-20]) but huge discrepancy occurred among three piroplasms genera, *Babesia*, *Theileria*, and *Cytauxzoon* [12]. Very recent study recognizes five distinct lineages of piroplasms based on congeniality of mitochondrial genome sequences concatenated with *18S* sequences [12]. (i) ***Babesia sensu stricto***, the lineage referring to organisms transovarially transmitted in ticks and reproducing exclusive in host erythrocytes (e.g. *Babesia bovis*, *Babesia bigemina*, *Babesia divergens*). (ii) ***Theileria* and *Cytauxzoon***, the lineage comprising organisms transmitted transstadialy in ticks and multiplicating in lymphocytes or monocytes prior the erythrocytes invasion (e.g. *Cytauxzoon felis*, *Theileria parva*, *Theileria annulata*). (iii) ***Theileria equi***, formerly referred as *Babesia equi* [21] but reclassified upon the description of multiplication in nucleated blood cells; the unique clade has been referred as sister clade of both *Babesia sensu stricto* and *Theileria* (+ *Cytauxzoon*) in other phylogenetic studies [5, 20]. (iv) **Western *Babesi*a group** represented by *Babesia conradae* (previously misattributed to *Babesia gibsoni* [22]) includes multiple organism identified in the Western US and recently in Africa and Europe (e.g. *Babesia duncani*, *Babesia lengau, Babesia behnkei*) [19, 20, 22-28]. (v) ***Babesia microti* group**, previously referred as Archaeopiroplasmida [19, 20, 29], which

includes an extensive complex of species (e.g. *Babesia vulpes*, *Babesia rhodaini*) that diverged early from the common ancestor of piroplasms.

Babesia, a worldwide distributed parasite of vertebrate animals

The *Babesia* parasite was discovered by Victor Babes, Romanian bacteriologist, who in 1888 firstly observed microorganisms inside bovine erythrocytes [30, 31]. The parasite was later identified as an agent of Texas Cattle Fever which turned out to be first description of an arthropod-transmitted pathogen of vertebrates (reviewed in [31]). Few years later, the first evidence of human infection by *Babesia* parasites was documented [32]. Since then, many of *Babesia* species have been documented and this number continues to increase, indicating potentially unsuspected high diversity of this group [33].

The cell of *Babesia* parasite is characterized by a unique collection of organelles – an apical complex – which gave a name to all Apicomplexan parasites. The complex is found in all invasive stages of the parasite and mediaties the attachment and penetration into the host cell [34]. The apical complex is located at the anterior end of the parasite cell and consists of skeletal and secretory components. Skeletal component consist of a conoid, polar rings and microtubular protrusions, and the secretory component of two to eight rhoptries, several dense granules and numerous micronemes [35-37]. In *Babesia* parasites, a few modifications of the apical complex occur. The species of the *Babesia sensu stricto* lineage lack the dense granule and instead possess an unique organelle, the spherical complex [34, 38, 39]. The apical complex of *B. microti* is far more reduced; it possesses single large rhoptry and lack the conoid, the polar rings and the microtubular part [40].

The host range of *Babesia* parasites is extremely wide and comprises hundreds of mammal hosts and also several avian animals. The complex of animal reservoirs and tick vectors makes *Babesia* one of the most common group of blood parasites worldwide (second after trypanosomes) [13]. Although the life cycle of many *Babesia* species has not been elucidated yet, the ticks of the family *Ixodidae* are the only known vectors (reviewed in [41]). To date, one possible exception was documented; the argasid soft tick *Ornithodorus erraticus* was suggested to transmit *Babesia meri*, a parasite of sand rats (*Psammomys obesus*) [42].

Babesia parasites multiply exclusively in erythrocytes of the vertebrate host and cause severe disease symptoms related to the host cells destruction. The asexual reproduction of *Babesia* in the host cells lacks the synchronicity. Thus, the trophozoites and merozoites of different phases of development occur in the blood stream at the same time. The disease is not accompanied by periodically relapsing symptoms as it is typical for malaria. Together with parasite asexual developmental stages, the first sexual stages – gametocytes – occur in the blood stream. Using the

light microscopy, the gametocytes are undistinguishable from merozoites. The sexual reproduction of *Babesia* parasite occurs in the gut lumen of the tick vector and starts with maturation of ingested gametocytes and production of gametes. Gametes – often named ray bodies or Strahlenkörper [2, 43] – of *Babesia* parasite are anisomorphic but the macro- and micro-gametes cannot be distinguished [44-47]. The gametes fusion gives rise to motile zygote penetrating the peritrophic membrane and invading gut epithelial cells. Here the parasite undergoes meiosis resulting in haploid motile kinetes, spreading through the tick internal organs including ovaries and salivary glands. Parasite is then transmitted either via larval progeny to eggs and larvae (transovarial transmission) or transstadially to molted stage of the tick vector. To finalize the development, the sporogony takes place in the salivary glands and fully matured sporozoites are produced, able to invade the erythrocytes of the host via tick bite [5, 6, 13, 16, 46-55]. The life cycle of *Babesia* parasites is more thoroughly described in the manuscript in preparation "Life cycle of piroplasms: comprehensive analysis". The unique adaptations in the development of *B. divergens* (Fig. 6) and *B. microti* (Fig. 11) are described further (Part III. and IV., respectively).

Babesiosis, a disease of veterinary and medical importance

Given by worldwide distribution of tick vectors, babesiosis belongs to the most common blood diseases of free living animals [13, 56] and is considered as an emergent zoonosis of humans [13, 56-59]. From the veterinary point of view, the great attention is paid to bovine babesiosis, the disease responsible for the enormous economic losses to the livestock industry [4] (see further). Nevertheless, babesiosis affects other animals and these diseases are often neglected. Equine babesiosis is the disease of horses and donkeys, caused by *Babesia caballi* (and *T. equi*), and the increasing densities of infected horses and ticks occur (reviewed in [7, 60]). The incidence of canine babesiosis is also on the rise and recently the dog diseases were reported from many countries worldwide (reviewed in [61]). The disease is caused by many *Babesia* species from *Babesia sensu stricto* lineage, Western *Babesia* lineage and *Babesia microti* lineage [12, 61].

Bovine babesiosis

Babesia parasite belongs to one of the most important arthropod-transmitted pathogen of cattle and the bovine disease is recognized for its major impact on farm animal health and connected with financial losses worldwide [4, 57, 62]. Bovine babesiosis, commonly called red water fever, is associated with mortalities, abortions, decreased meat as well as milk production and despite permanent epidemiologic surveillance the most of the 1-2 billion cattle around the world are still exposed to babesiosis and outbreaks occur [4, 62]. Disease symptoms include body weakness,

anemia, anorexia, diarrhea, high fever and hemoglobinuria (hence red water fever) but the disease manifestation can vary in dependence of the animal immune status, the number of questing ticks and parasite species and/or strain virulence [4, 57, 62, 63].

Four bovine *Babesia* species are recognized, *B. bovis*, *B. bigemina*, *B. divergens* and *Babesia major*. The worldwide distribution of these species is given by the tick vectors, responsible for spreading of the disease between the cattle. In tropical and subtropical areas of Australia, Africa, Asia and the Americas, the *B. bovis* and *B. bigemina* are found and transmitted by *Rhipicephalus* spp. ticks. On the contrary, in Europe the disease is caused by *B. divergens* and *B. major*. The bovine babesiosis caused by *B. divergens* is far the more common and reported across many countries (reviewed in [57]).

Human babesiosis

Humans are not natural hosts for any species of *Babesia* but serve as accidental hosts (reviewed e.g. in [41]). Despite this fact, the incidence of human babesiosis is on the rise as clinical cases are recently reported from many countries worldwide (reviewed e.g. in [6, 41]). Currently, human babesiosis is considered as an emerging tick-borne disease [59]. The first report of fatal human disease caused by *Babesia* parasite was documented in 1956 in patient from former Yugoslavia; the parasite was identified as *B. divergens* [64]. Since then, *B. divergens* was considered as the main European causative agent of human babesiosis and to date more than 30 cases of fatal disease were reported [6, 55, 65]. On the contrary, in USA the majority of the human diseases is attributed to the *B. microti* which is considered as major transfusion threat [6, 41, 58, 66] . Occasional cases caused by other *Babesia* species have also been described. In Europe the disease can be caused by *Babesia venatorum* as was reported in Austria, Italy and Germany [67, 68], and in USA by *B. duncani* [27, 69]. Cases of human babesiosis have also been reported in Africa, Asia, Australia and South America (reviewed e.g. in [6, 41, 55, 70].

The disease is usually transmitted during the tick (mostly *Ixodes* spp. [55]) bite but in rare cases babesiosis may be transmitted also by blood transfusion [5, 6, 56, 66, 69]. Babesiosis is frequently mistaken for malaria disease due to mimicry of somatic symptoms in the acute phase but lacks the typical periodicity. Most of healthy individuals suffer from flu-like symptoms and recover completely from babesiosis over four or more weeks (reviewed e.g. in [6, 13, 55, 70-72]). The severe and chronic infection generally occurs in people with immunosuppressive medication [68, 73], in malignancy [73], after splenectomy [73-75] or with HIV coinfection [76-78]. Among vulnerable groups belong also the elderly or very young people [6, 55, 72] and pregnant women [79]. Interestingly, *B. divergens* appears to cause more severe disease than *B. microti* [55, 57, 65] and the more severe illness occurs in patients co-suffering from Lyme disease as well [80, 81]. The disease is most often manifested by

fatigue, malaise, loss of appetite, weight loss, high fever or chills in the contrary accompanied with laboratory findings of anemia, disorders of blood count and elevation of liver enzymes [82, 83]. Also hematuria and hemoglobinuria can occur in more serious cases [13]. Human babesiosis therapy is standardly carried out by a combination of Azythromycine and Atovaquone [55, 84]. Dosage is related to the severity of the infection, the patient's condition and parasitemia, which normally ranges between 1 – 20% [59], but can reach up to 80% in particularly severe cases [85, 86]. There is no anti-babesiosis vaccine for human usage. The only way to prevent *Babesia* infection is to avoid areas with high occurrence of ticks or to early eliminate the tick.

Ticks as vectors of *Babesia* parasite

Six of the seven genera of hard ticks were demonstrated as experimental or natural vectors of various *Babesia* species [13]. The species used in the thesis, *B. divergens* and *B. microti*, are the species primarily transmitted by *Ixodes* spp. ticks. In Europe, the transmission of both species is mediated by *Ixodes ricinus*, the tick responsible for spread of bovine as well as human diseases [87]. In the thesis*, I. ricinus* represents a model vector in the performed experiments. Therefore, I further focus only on that species, neglecting other tick vectors of *Babesia* parasites.

Ixodes ricinus, the main European vector of *Babesia* parasites

The wide distribution area and a variety of vertebrate hosts make *I. ricinus* tick one of the most important vector of *Babesia* parasite. Actually, the incidence of babesiosis is attributed to distribution and density of *I. ricinus* in the European countries [6, 13, 55, 57, 62, 65]. *I. ricinus* serves as main vector of *B. divergens*, the most important agent of bovine babesiosis [57] and recently has been identified as the primary vector of *B. venatorum* (also reported as *Babesia* sp. EU1) [88-92]. The *I. ricinus* has been identified also as a competent vector of *Babesia capreoli* [93] and *B. microti* [94]. Currently, the *B. microti* infected *I. ricinus* ticks are reported from many European countries [95-109].

The hard tick *I. ricinus* is a three-host tick with a life cycle (Fig. 1) duration of 2-6 years; under favourable conditions in the natural environment the life cycle can be completed in one year. The prolongation of tick life cycle is a result of their adaptation long-term starvation which tick can survive for several years in adverse conditions. *I. ricinus* inhabits relatively humid areas such as woodlands, forests or heaths, where it can be found from early spring until late fall. The highest abundance can be monitored in the spring and autumn with a decline during the summer. Besides Europe, *I. ricinus* occurs in surrounding areas of the Middle East and North Africa [87].

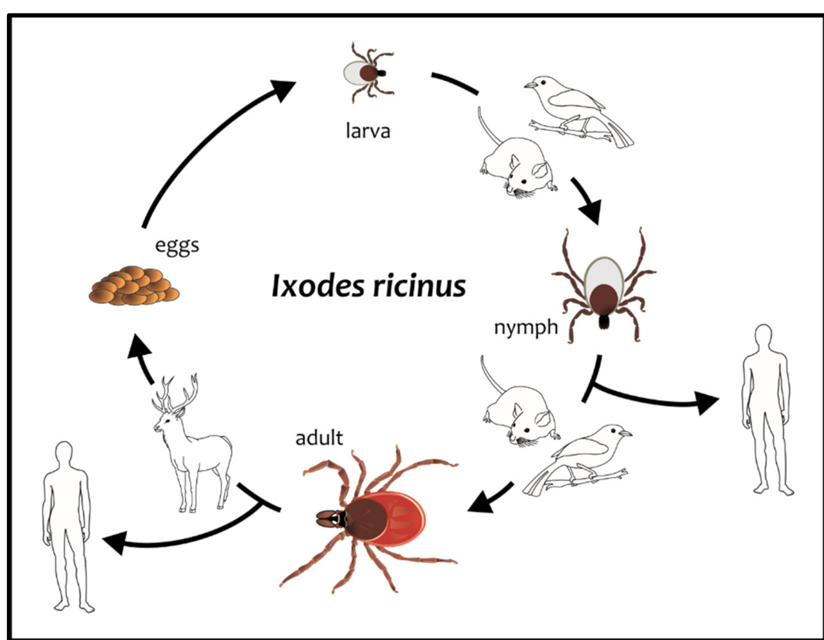

Figure 1. Life cycle of *I. ricinus*, the most common tick in Europe. Six legged larva is hatched from eggs and subsequently feed usually on the small vertebrates, mainly on rodents. After repletion and followed molting the eight legged nymphs appear and feed on middle sized animals like rodents or birds. After engorgement the nymphs turn into adults, males and females. Females then usually suck on the bigger vertebrates, such as deer, sheep or cattle. Fully engorged adult female's oviposition results into several thousands of spherical eggs. Figure was adapted from the illustration designed by M. Hajduskova (www.biographix.cz).

Mutual interaction of *Babesia* parasite and tick vector

Since tick serves as a host and vector of *Babesia* species, the parasite undergoes complicated developmental procedures in the tick internal organs. Therefore, the interaction between the *Babesia* parasite and tick vector occur and mediate their mutual relationship. In effort to map these interactions, we published a review "Interaction of the tick immune system with transmitted pathogens" [1] summarizing the knowledge about the problematics. Since the review was published, only few studies focused on the tick immune molecules mediating interaction with *Babesia* parasite. Several candidate molecules here have been identified *in silico* by gut transcriptome analysis of *Rhipicephalus microplus* upon the *B. bovis* infection [110] but results have not been verified by functional analyses yet. Merino and his colleagues attempted to confirm a previously identified promising anti-babesial tick proteins TROSPA [111] and subolesin [112], and showed that experimental vaccination of cattle with both proteins were able to reduce *B. bigemina* DNA levels in ticks fed on vaccinated cattle [113]. On the other hand, the potential anti-babesial function of candidate protein calreticulin [111] was excluded [114]. Nonetheless, no more studies about the problematics have been conducted to date.

Publication "Interaction of the tick immune system with transmitted pathogens"

The review (pages 9-23) summarizes the current knowledge of tick molecules potentially involved in interaction with the three intensively studied tick-transmitted pathogens – *Borrelia*, *Anaplasma* and *Babesia* – in context of their development in the tissues of vector ticks. I was involved in the review of tick molecules interacting with *Babesia* parasite.

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Interaction of the tick immune system with transmitted pathogens

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Ticks are hematophagous arachnids transmitting a wide variety of pathogens including viruses, bacteria, and protozoans to their vertebrate hosts. The tick vector competence has to be intimately linked to the ability of transmitted pathogens to evade tick defense mechanisms encountered on their route through the tick body comprising midgut, hemolymph, salivary glands or ovaries. Tick innate immunity is, like in other invertebrates, based on an orchestrated action of humoral and cellular immune responses. The direct antimicrobial defense in ticks is accomplished by a variety of small molecules such as defensins, lysozymes or by tick-specific antimicrobial compounds such as microplusin/hebraein or 5.3-kDa family proteins. Phagocytosis of the invading microbes by tick hemocytes is likely mediated by the primordial complement-like system composed of thioester-containing proteins, fibrinogen-related lectins and convertase-like factors. Moreover, an important role in survival of the ingested microbes seems to be played by host proteins and redox balance maintenance in the tick midgut. Here, we summarize recent knowledge about the major components of tick immune system and focus on their interaction with the relevant tick-transmitted pathogens, represented by spirochetes (*Borrelia*), rickettsiae (*Anaplasma*), and protozoans (*Babesia*). Availability of the tick genomic database and feasibility of functional genomics based on RNA interference greatly contribute to the understanding of molecular and cellular interplay at the tick-pathogen interface and may provide new targets for blocking the transmission of tick pathogens.

Keywords: tick, tick-borne diseases, innate immunity, phagocytosis, antimicrobial peptides, *Borrelia, Anaplasma, Babesia*

TICK-PATHOGEN INTERFACE: GENERAL CONSIDERATIONS

Ticks are the most versatile arthropod diseases vectors capable to transmit the broadest spectrum of pathogens comprising viruses, bacteria, protozoa, fungi and nematodes to their vertebrate hosts (Jongejan and Uilenberg, 2004). The tick-borne diseases, such as Lyme disease, tick-borne encephalitis, rickettsiosis (spotted fever), ehrlichiosis or human granulocytic anaplasmosis, are of great concern in human health and their serious threat discourage people from outdoor work or leisure activities. No less important are tick-transmitted zoonoses, such as anaplasmosis, babesiosis, theileriosis and African swine fever that cause substantial economic losses to the livestock production worldwide.

The success rate of pathogens transmitted by ticks is mainly given by the favorable aspects of tick physiology arising from their adaptation to the relatively long-lasting blood feeding. The modulation of host immune and inflammatory responses by various bioactive molecules present in the tick saliva (Francischetti et al., 2009) facilitates pathogen acquisition and transmission. Furthermore, the long-term persistence of ingested microbes in the midgut lumen is facilitated by the absence of extracellular digestive enzymatic apparatus, which is in ticks located inside the digestive vesicles of midgut cells (Sonenshine, 1991; Sojka et al., 2013). Nevertheless, ticks possess defense mechanisms that allow them to maintain the pathogens and commensal microbes at the level, which does not impair their fitness and further development. The long lasting co-evolution of ticks with pathogens resulted in the mutual tolerance, apparently adapted to the tick physiological differences (Mans, 2011). Therefore, the detailed knowledge of tick physiology and behavior is crucial to understand the fate of pathogens within the tick vector. For instance, the length of feeding, that strikingly differs between the hard and soft ticks (days vs. minutes, respectively), definitely shape the course of pathogen transmission. Pathogens transmitted by the hard ticks (*Ixodidae*) usually undergo several days of development until they infect the host. On the contrary, pathogens transmitted by the soft ticks (*Argasidae*) are ready for transmission immediately after the feeding starts. A good example here

is difference in the time of transmission between the *Borrelia* spirochetes causing Lyme disease (transmission several days after attachment) and relapsing fever (transmission several minutes after attachment) vectored by the hard and soft ticks, respectively (Sonenshine, 1991). Another important aspect that should be taken into consideration is the tick feeding strategy, the differences between one- and multi-host ticks in terms of transovarial and transstadial transmission.

The transmitting pathogen acquired from the infected host has to overcome several tissue barriers within the tick body comprising midgut, hemocoel and salivary glands or ovary (in case of transovarial transmission). Each of these compartments may play a decisive role in the tick vector competence for a certain microbe. The tick midgut is probably the most important tissue for survival and proliferation of the pathogens since many of them have to persist here until the molting and subsequent feeding. On their route from the midgut to the peripheral tissues, the pathogens are facing cellular and humoral defense mechanisms functioning within the tick hemolymph. Therefore, the abilities to cope with or avoid the tick immune responses are crucial for the pathogen transmission. During the last two decades, our knowledge about the invertebrate immunity has rapidly expanded, mainly given by the research on the model organisms such as fruit fly *Drosophila melanogaster* (Ferrandon et al., 2007), horseshoe crab, crayfish or ascidians (Iwanaga and Lee, 2005; Söderhäll, 2010). A substantial progress has been also made in the field of blood feeders, such as mosquitoes (Osta et al., 2004; Hillyer, 2010) and tsetse flies (Lehane et al., 2004). The information on the tick innate immunity is rather fragmentary and allows only approximate comparison with other invertebrates (Sonenshine and Hynes, 2008; Kopacek et al., 2010). Nevertheless, even these scattered data indicate that ticks possess defense mechanisms protecting them against microbial infection (**Figure 1**). At the cellular level, they comprise phagocytosis, encapsulation and nodulation of foreign elements. The humoral defense is based on a variety of pattern-recognition proteins and effector molecules such as lectins, complement-related molecules and a broad spectrum of common as well as specific antimicrobial peptides (AMPs) (Kopacek et al., 2010). In addition, possibly important but rather unexplored role in the tick defense system is played by the immune molecules of the host origin.

In this review, we will follow the transmission routes of pathogens and subsequently enumerate the potential obstacles they have to evade in the tick body. The general features of tick immunity will be further discussed in relation to our current knowledge of tick interaction with the three most intensively studied agents of tick-borne diseases, represented here by *Borrelia* spirochete, intracellular rickettsia *Anaplasma*, and malaria-like protozoa *Babesia*.

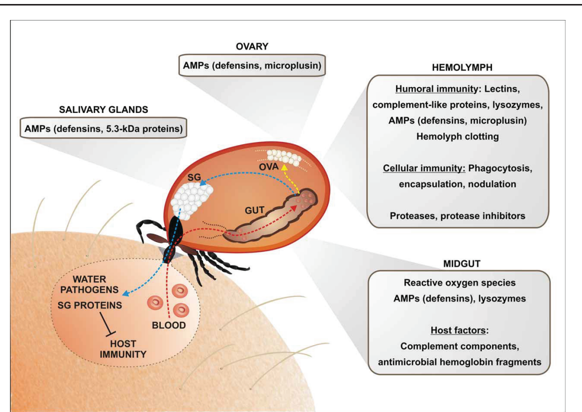

FIGURE 1 | An overview of the tick immune mechanisms and molecules constituting potential barriers for the pathogen transmission. The pathogen transmission is tightly linked with physiology of blood feeding and tick innate immunity. Ingested blood meal is accumulated in the midgut content (red arrow; only one caecum shown). Hemoglobin and other proteins are taken up by the tick midgut cells and digested intracellularly in the lysosome-like digestive vesicles (Sojka et al., 2013). Liberated amino acids and other compounds are transported to the peripheral tissues and ovaries, supplying mainly egg development (yellow arrow). Importantly, the blood meal is concentrated by reabsorption of excessive water, which is spitted back into the wound by the action of salivary glands (blue arrow). Tick saliva contains a great variety of anti-coagulant, immunomodulatory and anti-inflammatory molecules that facilitate pathogen acquisition and transmission. The ingested pathogens have to survive the period between detachment and subsequent feeding of the next tick developmental stage and overcome several obstacles on its route through the tick body. In the midgut, tick may utilize some of the host immune molecules (e.g., complement system) for its own defense against intestinal inhabitants. Hemoglobin fragments, derived from the host hemoglobin, are secreted into the midgut lumen and exert strong antimicrobial activity. Tick midgut tissue also expresses a variety of endogenous AMPs, which sustain the midgut microbes at a tolerable level. An important, but still poorly understood, role is most likely played by the maintenance of the redox homeostasis in the tick midgut. Pathogens intruding into the tick hemocoel can be phagocytosed by tick hemocytes or destroyed by effector molecules of the humoral defense system, comprising AMPs, components of the primordial complement system (thioester-containing proteins (TEPs), convertase-like factors and fibrinogen-related lectins (FREPs). Ticks probably possess a mechanism of hemolymph clotting, but genes/proteins putatively involved in the activation of prophenoloxidase cascade leading to melanization have not yet been identified in any tick species. Tick salivary glands express also a variety of AMPs, which may impair pathogen acquisition and persistence in the tick, as demonstrated for the 5.3-kDa antimicrobial peptides and their role in the defense against *Anaplasma* infection (Liu et al., 2012). Abbreviations: GUT, midgut; OVA, Ovary; SG, salivary glands.

TICK IMMUNE SYSTEM

TICK MIDGUT—THE PRIMARY SITE OF TICK-PATHOGEN INTERACTIONS

Although the midgut of arthropod disease vectors is most likely the principle organ that determines their vector competence, the general knowledge of the mutual interplay between ingested pathogen, commensal microflora and tick itself is still inadequate. Unlike in mosquitoes and other insect blood feeders, the microbes ingested by ticks are not in direct contact with digestive proteases secreted into the lumen and the highly nutritious broth of concentrated blood proteins, neutral pH and long-term storage present an ideal environment for microbial proliferation. Therefore, ticks have to possess efficient defense mechanisms which maintain the intestinal microflora at tolerable level.

Two recent high-throughput mapping projects of the microflora (microbiome) by the next generation sequencing were carried out in two tick species, *R. microplus* (Andreotti et al., 2011) and *Ixodes ricinus* (Carpi et al., 2011). These studies revealed an extreme diversity of the bacterial community (more than hundred different organisms identified in one tick species), which apparently reflects tick geographical and environmental origin as well as developmental stage. However, encounter with a microbe, which a tick hardly meets in nature, could have a fatal consequence because of the lack of effective defense. A good example is the artificial infection of soft tick, *Ornithodoros moubata*, with the Gram (−) bacterium, *Chryseobacterium indologenes* (Buresova et al., 2006), which resulted in rapid tick death. Although this soft tick secretes into the midgut lumen at least two kinds of antimicrobial compounds protecting against Gram (+) bacteria, lysozyme (Kopacek et al., 1999; Grunclova et al., 2003) and defensins (Nakajima et al., 2001, 2002), these molecules apparently fail to protect the ticks against some Gram (−) bacteria. Defensins have been also frequently reported to be expressed in the midgut tissues of hard ticks (Hynes et al., 2005; Rudenko et al., 2005; Zhou et al., 2007), but their secretion and antimicrobial activity in the midgut lumen has not yet been unambiguously demonstrated. A defensin-related molecule named longicin, expressed in the midgut of *Haemaphysalis longicornis*, was reported to be active against a variety of microbes including Gram (+) and Gram (−) bacteria, fungi and various *Babesia* species (Tsuji et al., 2007) (see also below).

A specific role of the midgut defense against Gram (+) and some fungi is played by the antimicrobial activity of large peptides derived from the host hemoglobin (hemocidins). The antibacterial hemoglobin fragments were initially isolated from the midgut contents of the cattle tick *R. microplus* (Fogaca et al., 1999) and later also identified in the midgut of other soft and hard tick species (Nakajima et al., 2003; Sonenshine et al., 2005). The generation of antimicrobial hemoglobin fragments most likely occurs in the digestive cells during the initial phase of hemoglobin digestion by the synergic action of cathepsin D-type and cathepsin L-type aspartic and cysteine peptidases, respectively (Horn et al., 2009; Cruz et al., 2010).

Hemoglobin digestion and the concomitant process of heme detoxification via hemosome formation (Lara et al., 2003) is necessarily associated with the maintenance of the redox homeostasis in the tick midgut. Although this process is virtually unknown in ticks, the paradigm to follow is the recent seminal finding on the importance of redox balance in the mosquito midgut epithelial immunity. In the malaria vector *Anopheles gambiae,* a tandem of heme peroxidase and dual oxidase (Duox) catalyzes formation of dityrosine network between the midgut epithelium and lumen. This network prevents delivery of the epithelial immunity elicitors and ultimately results in up-regulation of intestinal microflora and *Plasmodium* infection in the lumen (Kumar et al., 2010). Heme peroxidase and NADPH oxidase 5 (Nox5) were further shown to mediate the epithelial nitration of *Plasmodium* ookinetes and hereby their opsonization for subsequent lysis by the complement-like action of thioester-containing protein TEP1 (Oliveira Gde et al., 2012). The redox situation may also indirectly affect the pathogen transmission by changing its balance with other microflora present in the midgut. An example, how the midgut microflora determines the competence of *A. gambiae* and malaria parasites was reported recently, showing that ROS produced by the mosquito midgut dweller *Enterobacter* sp. interfere with *Plasmodium* development (Cirimotich et al., 2011). The interrelationship between the redox balance and intestinal microflora could be quite complex, as demonstrated using sugar vs. blood fed mosquitoes *Aedes aegypti* (Oliveira et al., 2011). The presence of heme in the mosquito diet caused a significant decrease of ROS levels, resulting in consequent expansion of midgut bacteria. This phenomenon was interpreted as a result of the mosquito adaptation against the high oxidative stress potentially caused by reaction of pro-oxidative heme with high levels of continuously produced ROS (Oliveira et al., 2011).

By contrast, very little is known about the maintenance of redox homeostasis in the tick midgut except for one report showing the role of catalase in the regulation of the oxidative stress in the cattle tick *R. microplus* (Citelli et al., 2007) and the seminal work on the heme-detoxification pathway described in the same species (Lara et al., 2003, 2005). Nevertheless, the genomic and transcriptomics data from other tick species suggest that ticks do maintain the redox homeostasis in their midguts as they possess ROS-generating enzymes, such as NOX5 or DUOX, and arsenal of antioxidant enzymes and radical scavengers comprising catalases, glutathione- and thioredoxin peroxidases, glutathione S-transferases, and selenoproteins (Anderson et al., 2008; Megy et al., 2012). Thus, the framework of redox balance and its direct or indirect impact on the persistence of pathogens in the tick midgut offers almost unlimited inspiration for the further research.

IMMUNE REACTIONS WITHIN THE TICK HEMOLYMPH

The major portion of our knowledge on the tick innate immunity is associated with cellular and humoral immune responses within the tick hemocoel. The volume of tick hemolymph increases linearly during the tick feeding from about 2–3 μl in unfed to almost 150 μl in fully engorged females, as demonstrated for *Dermacentor andersoni* (Kaufman and Phillips, 1973). At least three types of hemocytes, namely plasmatocytes, granulocytes I and granulocytes II, have been recognized in the hard and soft ticks, out of which the former two are phagocytic (Sonenshine, 1991; Borovickova and Hypsa, 2005). Several studies demonstrated the capability of hemocytes from different tick species to engulf foreign material and different microbes (Inoue et al., 2001; Loosova et al., 2001; Buresova et al., 2006). In addition, it was demonstrated that phagocytosis of microbes by the tick hemocytes is associated with humoral defense mechanisms, such as the production of ROS (Pereira et al., 2001) or complement-like molecules (Buresova et al., 2009, 2011). The process of hemocytic encapsulation of artificial implants, possibly linked with hemolymph coagulation and cellular response against *Escherichia coli* resembling nodulation, was reported to occur in the hemocoel of *Dermacentor variabilis* (Eggenberger et al., 1990; Ceraul et al., 2002).

Of special interest is the phagocytosis of tick-transmitted pathogens, such as *Borrelia* spirochetes, which seem to be

engulfed at least in part by the process of "coiling" phagocytosis (Rittig et al., 1996). A comparison of the phagocytic and borreliacidal activity against *B. burgdorferi* injected into the hemocoel of natural vector, *I. scapularis* and a refractory tick *D. variabilis*, revealed much stronger immune response against the spirochetes in the latter immunocompetent tick species (Johns et al., 2000, 2001a). On the other hand, it was recently shown that infection of *I. scapularis* hemocytes by *A. phagocytophilum* is mediated by the protein named P11 and is required for successful migration of the pathogen from the midgut to salivary glands (Liu et al., 2011), meaning that phagocytosis or engulfment of the pathogen by tick hemocytes does not necessarily cause its elimination. This rise an interesting question whether at least some of the tick-transmitted pathogens may take an advantage of being engulfed by tick hemocytes to hide from the attack of humoral immune responses in the hemocoel.

Effector molecules of several types have been described in the tick hemolymph, out of which reports on tick defensins are the most frequent since they have been identified in a number of hard and soft tick species (Chrudimska et al., 2010; Kopacek et al., 2010). Moreover, the recent analysis of *I. scapularis* genome revealed an extensive expansion of genes encoding for defensins and defensin-like peptides divided into two multi-gene families referred to as scapularisins and scasins, respectively (Wang and Zhu, 2011). Typical mature defensins are $\sim$4 kDa cationic peptides with a conserved pattern of six paired cysteins, derived by C-terminal cleavage after the furin (RVVR) motif from $\sim$8 kDa pre-prodefensin. Tick defensins are usually active against Gram (+) bacteria and their interactions with transmitted pathogens [except for the above mentioned longicin (Tsuji et al., 2007)] have not been yet unequivocally demonstrated. Varisin, a defensin isolated from the hemolymph of *D. variabilis*, exerted a borreliacidal effect in combination with lysozyme (but not alone), which may in part explain the incompetence of this species to sustain *B. burgdorferi* spirochetes (Johns et al., 2001b). Interestingly, depletion of varisin from the *D. variabilis* hemolymph using RNA interference resulted in the significant reduction of *Anaplasma marginale* infection, indicating that the impact of defense mechanisms on a certain pathogen might be quite complex and not always predictable (Kocan et al., 2008b, 2009).

In addition to defensins, ticks possess a specific class of histidine- and cysteine-rich antimicrobial peptides of size about 10 kDa, namely hebraein identified in *Amblyoma hebraeum* (Lai et al., 2004) and microplusin isolated from the hemolymph of *R. microplus* (Fogaca et al., 2004). Unlike defensins, which kill bacteria in a detergent-like manner by disruption of bacterial membranes, the bacteriostatic effect of microplusin is based on its capacity to sequester copper required mainly for bacterial respiration (Silva et al., 2009). Another cysteine-rich antimicrobial peptide, unrelated to microplusin and referred to as ixodidin, was isolated from *R. microplus* hemocytes and its antibacterial activity was proposed to be linked to the inhibitory activity against serine proteases by yet unknown mechanism (Fogaca et al., 2006).

The process of self/nonself recognition within the tick hemolymph is believed to involve the interaction of tick lectins and carbohydrates associated with the invading microbes (PAMPs or pathogen-associated molecular patterns). The activity of lectins/hemagglutinins with preferential binding specificity for N-acetyl-D-hexosamines, sialic acids and glycoconjugates have been identified in the hemolymph of several hard and soft tick species (Grubhoffer et al., 2008; Sterba et al., 2011) and is mainly attributed to the presence of fibrinogen-related proteins (FREPs) related to Dorin M, isolated and characterized from the soft tick *O. moubata* (Kovar et al., 2000; Rego et al., 2006). In contrast to mammalian ficolins, Dorin M lacks the N-terminal collagen-like domain and is closely related to the lectins of tachylectin-5 type known to function as pattern recognition molecules in the horseshoe crab immune system (Gokudan et al., 1999; Kawabata and Tsuda, 2002; Ng et al., 2007). The genomes of *I. scapularis* and *I. ricinus* contain genes encoding for a variety of FREPs named Ixoderins that can be phylogenetically divided into three major groups (Rego et al., 2005; Kopacek et al., 2010). Although the role of FREP family members in the invertebrate immunity may be multifunctional, as recently suggested for gastropod mollusk (Hanington and Zhang, 2011), we hypothesize that at least some tick FREPs play a role in activation of tick complement system, components of which have been identified in ticks (Kopacek et al., 2012).

Ticks are unique among other invertebrates in that they possess representatives of all major classes of thioester-containing proteins (TEP) known in vertebrates and arthropods: (1) molecules related to α2-macroglobulins, (2) C3-components of complement system and (3) insect TEPs and (4) macroglobulin complement-related proteins (MCR) (Buresova et al., 2006). The pan protease inhibitors of α_2-macroglobulin type were reported to be present in the hemolymph of soft and hard ticks (Kopacek et al., 2000; Saravanan et al., 2003; Buresova et al., 2009), where they presumably protect the ticks against undesired proteolytic attack of endogenous as well as exogenous proteases including those of invading microbes. The inhibition of metalloproteases secreted by the Gram (−) bacterium *C. indologenes* was shown to be functionally linked with phagocytosis of this bacteria by the tick hemocytes (Buresova et al., 2009). Further functional study of the tick TEPs suggested that phagocytosis of different bacteria by the tick hemocytes depends on non-redundant involvement of various tick TEPs with a central role of C3-like molecules (Buresova et al., 2011). Although nothing is known about interaction of the tick TEPs with tick-transmitted pathogens, the paradigm of *A. gambiae* TEP1 as a complement-like molecule, which determines the mosquito competence to *Plasmodium* parasites (Blandin et al., 2004, 2008), should stimulate further research in this area. In addition to the TEP family, genome of *I. scapularis* contains genes encoding for putative C3 convertases (Kopacek et al., 2012) having the multi-domain architecture similar to that of factor C2/Bf and LPS-sensitive Factor C activating the ancient complement-like system in the horseshoe crab (Zhu et al., 2005; Ariki et al., 2008). These preliminary results suggest that ticks possess features of a primitive complement system, which evolved on Earth at least one billion years ago (Nonaka and Kimura, 2006).

The existence of tick molecule related to the horseshoe crab Factor C, which primarily serves to trigger the limulus clotting cascade upon recognition of bacterial endotoxins (Kawabata,

2010), may suggest that ticks possess also a system for hemolymph coagulation. This suggestion was in part corroborated by the high throughput screening of immune-responsive genes in *D. variabilis* challenged with different bacteria. The most inducible immune gene found among others was transglutaminase, which acts as a crosslinking enzyme in the terminal phase of clotting mesh formation (Jaworski et al., 2010). However, with a possible exception of the previous observation, where a fibrous matrix was formed around the Epon-Araldite particles implanted under *D. variabilis* cuticle (Eggenberger et al., 1990), a convincing evidence of hemolymph clotting in ticks is still missing.

In contrast to other arthropods, ticks most likely lack the prophenoloxidase (PPO) activation system leading to melanization, because no PPO-related gene has been yet identified neither in the genome of *I. scapularis* (Megy et al., 2012) nor within the extensive EST datasets from other tick species (Kopacek et al., 2010).

IMMUNE REACTIONS WITHIN THE SALIVARY GLANDS

The tick salivary glands and components of tick saliva have been investigated foremost for their indispensable role in the modulation of host hemostasis, inflammation and immune response at the tick-host interface (Francischetti et al., 2009). The increasing number of salivary glands transcriptomes (sialomes) from the hard and soft ticks revealed the expression of a various AMPs, such as defensins, microplusin/hebraein and lysozymes, in this tissue (Mans et al., 2008; Karim et al., 2011). A defensin-like peptide named longicornisin was purified from the salivary glands of *H. longicornis* (Lu et al., 2010) and two different antimicrobial peptides unrelated to any known AMPs designated as Ixosin and Ixosin B were isolated from the salivary glands of *Ixodes sinensis* (Yu et al., 2006; Liu et al., 2008). However, it still has not been demonstrated whether these salivary glands AMPs are secreted into the tick saliva or hemolymph and if they directly interact with pathogens. The only exception is the 5.3-kDa antimicrobial protein, referred to as ISAMP and isolated from the saliva of *I. scapularis*, which exerts activity against Gram (−) and Gram (+) bacteria (Pichu et al., 2009). The transcripts encoding the family of secreted 5.3-kDa proteins were previously described to be significantly enriched in the transcriptome of *I. scapularis* nymphs infected with *B. burgdorferi* (Ribeiro et al., 2006). More recently, it was demonstrated that the 5.3-kDa family members were markedly upregulated in the salivary glands and hemocytes during *A. phagocytophilum* infection and were involved in the *I. scapularis* defense against this pathogen. Intriguingly, they were also shown to be effector molecules regulated by the JAK-STAT pathway (Liu et al., 2012) and although the *I. scapularis* genome contains also components of the putative Toll and Imd immune signaling pathways (Megy et al., 2012; Severo et al., 2013), the 5.3-kDa family regulation by JAK/STAT is the only so far described case of tick antimicrobial response controlled by a signaling pathway.

RNA INTERFERENCE—AN ANTIVIRAL DEFENSE IN TICKS

The RNA interference (RNAi) is an ancient mechanism evolved for the inhibition of foreign genetic elements and precise regulation of the endogenous genes during organism development (Myers and Ferrell, 2005). The RNAi seems to work very well in the tick tissues (De La Fuente et al., 2007b) and the genome of *I. scapularis* contains all components important for the endogenous and exogenous RNAi machinery including dicers, argonauts, dsRNA binding proteins, exonucleases and surprisingly also RNA-dependent RNA polymerases (Kurscheid et al., 2009). The discovery that plant viruses encoded suppressors of the gene silencing machinery provided a strong support for RNAi function as a natural defense mechanism against viruses (Lindbo et al., 1993; Ratcliff et al., 1999). It was shown that viral proteins identified as suppressors in plants and insect cells were able to abrogate RNA silencing also in the tick cells (Garcia et al., 2006). In the context of tick immunity, we can speculate that RNAi could interfere directly with the viral infection or regulate production of antimicrobial peptides through the expression of microRNAs.

TICK INTERACTIONS WITH TRANSMITTED PATHOGENS

BORRELIA

Lyme disease is an emerging human tick-borne disease of temperate climates with a concurrent distribution spanning North America and Eurasia. It is caused by *Borrelia* spirochetes related to *Treponema* and *Leptospira*, mainly by *Borrelia burgdorferi* sensu stricto in the US. and *B. burgdorferi* sensu stricto, *B. garinii*, and *B. afzelii* in Europe (Radolf and Samuels, 2010). Borreliosis in humans affects multiple body systems, producing a range of potential symptoms (Burgdorfer et al., 1989). The classical sign of early infection is circular, expanding, skin rash at the tick bite site called *erythema migrans*. Treatment with antibiotics is effective at this stage of infection. When left untreated, the spirochetes disseminate throughout the body and are associated with arthritis (*B. burgdorferi* sensu stricto), neurological symptoms (*B. garinii*) or dermatitis (*B. afzelii*) (Stanek et al., 2012). Although Lyme disease is intensively studied, an effective vaccine is still not available and annual incidence in many countries continues leading over other human vector-borne diseases (Bacon et al., 2008).

Borrelia spirochetes survive in an enzootic cycle involving three-host *Ixodes* ticks and small animals like rodents, birds and lizards (Steere et al., 2004). The spirochetes are usually not detected in larger mammals, which are essential in the tick life cycle as a source of sufficient amount of blood for feeding females (adult female of *Ixodes* ticks can take in total about one milliliter of blood Balashov, 1972), but complement system of the vertebrate innate immunity lyses most of the bacteria (De Taeye et al., 2013). Humans are not able to efficiently kill the pathogens and often get infected. However, they are mostly dead-end hosts for both the ticks and the pathogens. Transovarial transmission of *Borrelia* in ticks is not likely (Rollend et al., 2013) and people can contract Lyme disease only by feeding of infected nymphs or adults, where nymphs play a key role in the epidemiology of disease because of their small size and relatively short feeding time.

The interplay between tick proteins, *Borrelia* spirochetes and hosts has been mapped by transcriptomics and proteomics studies (Narasimhan et al., 2007a), antibody-screening assays (Das et al., 2001) and yeast-surface displays (Schuijt et al., 2011b). Several tick genes have been identified as crucial for acquisition of the infection in ticks, *Borrelia* persistence in the midgut

and transmission into the next host during subsequent feeding (**Figure 2** and **Table 1**). *Borrelia* colonization of the tick midgut lumen and their persistence until the next feeding is crucial process for the successful transmission of the parasite. *Borrelia* outer surface protein A (OspA) (De Silva et al., 1996), which is expressed predominantly inside the tick vector, is essential for pathogen adherence to the midgut cells during the acquisition phase and plays a significant role in the pathogen persistence. During the subsequent feeding, OspA expression is suppressed, but upregulated expression of OspC facilitates invasion of the tick salivary glands and transmission to the new host (Schwan et al., 1995). Tick protein called TROSPA (tick receptor for OspA) has been found to be implicated in the binding of OspA (Pal et al., 2004). TROSPA is specifically expressed in the midgut and its mRNA levels increase following the spirochete infection and decrease in response to engorgement. Importantly, interference with TROSPA expression by RNAi or its saturation by TROSPA antisera reduces *Borrelia* adherence to the midgut surface, preventing pathogen colonization of the vector and reducing its transmission (Pal et al., 2004).

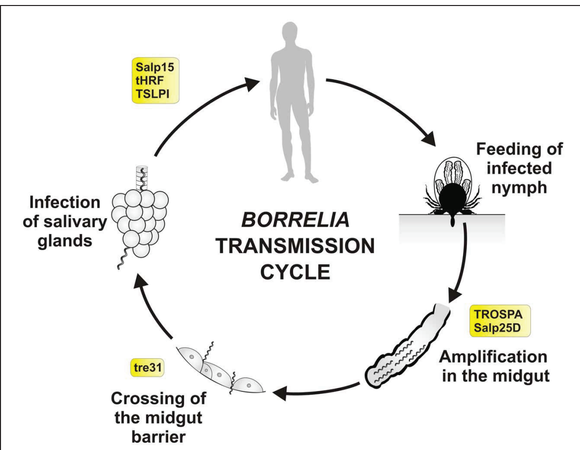

FIGURE 2 | Tick molecules involved in *Borrelia* transmission. Schematic diagram representing general stages of *B. burgdorferi* sensu lato infection and transmission in the *Ixodes* nymph, the most important developmental stage for human infection. Tick molecules, for which interference with *Borrelia* acquisition, persistence and/or transmission have been proved by genetic tests, are shown in rectangles (see text and the **Table 1** for their function and references). *Borrelia* spirochetes, that persisted in the tick midgut after the previous feeding (transstadial transmission), multiply rapidly within the newly engorged blood. They remain immobile and attached to the midgut cells. Around 53–72 h after the placement spirochetes become motile and swiftly cross the midgut barrier (between the cells), enter the hemolymph, salivary glands and via the saliva they infect new host (Ohnishi et al., 2001; Dunham-Ems et al., 2009). Transovarial transmission of *Borrelia* does not occur and newly hatched larvae are not infectious.

An antibody-screening assay performed on rabbit sera with acquired resistance to the tick bites after *I. scapularis* infestation identified salivary gland protein called Salp25D (Das et al., 2001). Salp25D encodes for a glutathione peroxidase, is upregulated upon feeding, and silencing of this gene by RNAi or immunization of mice with the recombinant protein impairs spirochete acquisition by ticks (Narasimhan et al., 2007b). Thus, Salp25D is most likely important for quenching the reactive oxygen species released from the activated neutrophils and hereby protects *Borrelia* during acquisition and colonization of the tick midgut.

During the tick feeding, *Borrelia* spirochetes, which multiplied previously in the midgut content, cross midgut barrier (between the cells) to get into the hemolymph and salivary glands (De Silva and Fikrig, 1995; Hojgaard et al., 2008; Dunham-Ems et al., 2009). *Borrelia* enolase, an enzyme found on the surface of spirochetes, was shown to bind host plasminogen and facilitate dissemination of *Borrelia* in the ticks and host (Coleman et al., 1995, 1997). In the later study, yeast surface display approach identified that *Borrelia* outer-surface lipoprotein BBE31 interacted with the tick protein called tre31 (Zhang et al., 2011). Expression of tre31 is induced in the midgut upon *Borrelia* infection and silencing of tre31 by RNAi or blocking of BBE31 using mice antibodies decreases spirochete burden in the hemolymph and salivary glands of feeding ticks.

It has been shown before that proteins contained in the tick saliva had strong pharmacological properties, targeting coagulation, platelet aggregation, vasoconstriction (Chmelar et al., 2012) and complement system (De Taeye et al., 2013). Salp15 is a salivary gland protein with remarkable immunosuppressive properties, which is bound by *Borrelia* OspC surface protein during host invasion and protects the spirochetes from antibody-mediated killing (Ramamoorthi et al., 2005). Its expression is upregulated in salivary glands upon *Borrelia* infection and silencing of Salp15 by RNAi dramatically reduces the capacity of spirochetes to infect mice. Moreover, antibodies raised against tick Salp15 protect

Table 1 | Tick molecules interfering with *Borrelia* acquisition, persistence and/or transmission.

Name	Supposed function	RNAi effect on the pathogen	References
Tick receptor for OspA (TROSPA)	Unknown	Reduced acquisition	Pal et al., 2004
Salivary protein 15 (Salp15)	Inhibition of the host complement system	Decreased transmission	Ramamoorthi et al., 2005
Salivary protein 25D (Salp25D)	Glutathione peroxidase	Decreased acquisition	Narasimhan et al., 2007b
Tick histamine release factor (tHFR)	Stimulation of histamine release	Decreased transmission	Dai et al., 2010
Tick receptor of BBE31 (tre31)	Unknown	Reduced persistence	Zhang et al., 2011
Tick salivary lectin pathway inhibitor (TSLPI, P8)	Inhibition of the host complement system	Decreased persistence and transmission	Schuijt et al., 2011a

mice from the infection (Dai et al., 2009). Tick salivary lectin pathway inhibitor TSLPI, previously identified by yeast surface display assay as P8 protein with ability to reduce complement killing of *Borrelia* (Schuijt et al., 2011b), interferes with lectin complement pathway, resulting in impaired neutrophil phagocytosis and chemotaxis (Schuijt et al., 2011a). Silencing of this protein by RNAi or exposure of ticks to TSLPI-immunized mice decreases persistence of *Borrelia* in nymphs and hampers their transmission, respectively. Tick histamine-release factor tHRF is a saliva protein able to bind host basophils and stimulate histamine release (Dai et al., 2010). This property can be exploited by *Borrelia* spirochetes for host infection. Expression of tHRF is upregulated in *Borrelia*-infected ticks and silencing of this gene by RNAi or tHRF blocking by antibodies reduce tick feeding and decrease spirochete burden in mice. The last molecule that should be mention is tick salivary protein named Salp20, which is an inhibitor of alternative complement pathway and partially protects serum sensitive species of *Borrelia* from lysis (Tyson et al., 2007) by displacing properdin from C3 convertase (Tyson et al., 2008). However, functional genetic studies are needed to prove its role *in vivo*.

ANAPLASMA

Anaplasmosis is considered as one of the most important vector-borne diseases of livestock (Kocan et al., 2010). The genus *Anaplasma* (Rickettsiales: Anaplasmataceae) includes six species of obligate intracellular bacteria, closely related to *Ehrlichia*, *Wohlbachia,* and *Neorickettsia*, occurring within the membrane-bound vacuoles called colonies in the host cytoplasm (Dumler et al., 2001; Kocan et al., 2008a). The *Anaplasma* rickettsiae preferably infect vertebrate red blood cells, however *A. phagocytophilum* attacks host neutrophils.

A. phagocytophilum infects a wide range of animals. It is responsible for the human granulocytic anaplasmosis (HGA), an emerging disease in the US, Europe and Asia, tick-borne fever in ruminants and equine and canine anaplasmosis (Woldehiwet, 2010). Three *Anaplasma* species exclusively infect ruminants: *A. marginale, A. centrale,* and *A. ovis*. *A. centrale* is used as life cattle vaccine in some regions, because infection with this parasite results only in mild clinical symptoms and could leave cattle persistently infected but immune against *A. marginale*, the causative agent of bovine anaplasmosis, which causes economic losses to the cattle industry worldwide. *A. ovis* is infective for sheep and wild ruminants, but infections are usually asymptomatic (Kocan et al., 2010). Also included in the genus *Anaplasma* are *A. bovis* and *A. platys*, which infect cattle and dogs, respectively.

All *Anaplasma* species are transmitted by Ixodid ticks, although tick transmissibility of *A. centrale* has been recently questioned (Shkap et al., 2009). The transmission cycle has been most extensively studied for *A. marginale* (Kocan et al., 1986, 1992a,b, 2008a). The developmental cycle in ticks is well coordinated with feeding and two *Anaplasma* morphotypes, reticulate (cell-dividing form) and dense core (infective form), can be found at each site of development (Kocan et al., 2010). Transovarial transmission of *Anaplasma* spp. from female ticks to their progeny does not occur. Therefore, ticks must acquire infection during blood feeding and the transmission cycles of these bacteria in nature are dependent upon the presence of infected reservoir hosts. Transmission by one-host ticks is probably accomplished by males, which can feed repeatedly and transfer between hosts (Sonenshine, 1991).

It has been shown that *Anaplasma* spp. modulate gene expression in ticks (De La Fuente et al., 2007a; Kocan et al., 2008a; Zivkovic et al., 2009; Sultana et al., 2010; Villar et al., 2010a,b), although differences may exist between species (Zivkovic et al., 2009). Functional studies of tick-*Anaplasma* interactions have shown how tick genes may affect bacterial infection (**Figure 3** and **Table 2**). Four differentially regulated genes/proteins, glutathione S-transferase (GST), salivary selenoprotein M (SelM), vATPase, and ubiquitin have been identified by suppression-subtractive hybridization and differential in-gel electrophoresis analyses using tick IDE8 cells infected with *A. marginale* (De La Fuente et al., 2007a). Glutathione S-transferases are intracellular enzymes with various functions, mostly accompanying cellular detoxification, but also signaling (Oakley, 2011). Selenoproteins are selenocysteine-containing proteins and important antioxidants (Reeves and Hoffmann, 2009). Vacuolar H^+ ATPases are membrane proteins acidifying a wide array of intracellular organelles by pumping protons across the plasma membranes (Nelson, 2003). Finally, ubiquitins are small regulatory proteins, involved

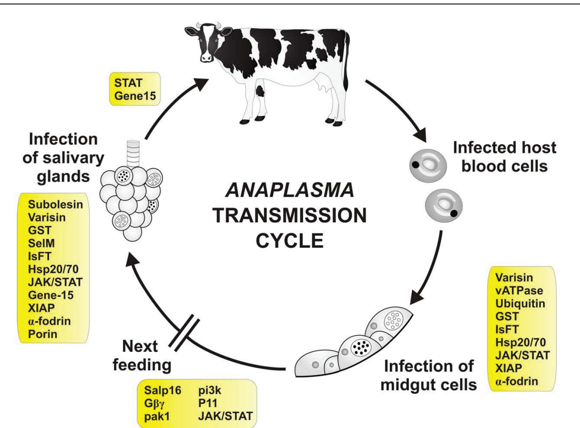

FIGURE 3 | Tick molecules involved in *Anaplasma* transmission. Schematic diagram representing general stages of *Anaplasma* infection and transmission (Kocan et al., 2008a). Tick genes, for which interference with *Anaplasma* acquisition and/or transmission have been proved by genetic tests, are shown in rectangles (see text and the **Table 2** for their function and references). Infected red blood cells (neutrophils for *A. phagocytophilum*) are engorged by the tick during blood meal. The released bacteria infect tick midgut cells and develop reticulate (cell-dividing; open circle) and dense core (infective; filled circle) forms of colonies inside the cells. During the next feeding, bacteria are released from the cells and infect other tissues including salivary glands. Here, they multiply inside the cells and are released into the saliva and transferred into the new host. Infection of tick hemocytes is required for the pathogen migration from the midgut to the salivary glands (Liu et al., 2011). Transovarial transmission of *Anaplasma* does not seem to occur. Transmission in one-host ticks is probably accompanied by tick males, which can feed repeatedly and transfer between hosts.

Table 2 | Tick molecules interfering with *Anaplasma* acquisition and/or transmission.

Name	Supposed function	RNAi effect on the pathogen	References
Salivary protein 16 (Salp16)	Unknown	Decreased acquisition	Sukumaran et al., 2006
Subolesin (SUB)	Component of the immune signaling pathways	Decreased acquisition	De La Fuente et al., 2006, 2008; Merino et al., 2011; Busby et al., 2012
Varisin	Defensin	Decreased acquisition	Kocan et al., 2008b
Vacuolar H^+ ATPase (vATPase)	Acidification of vesicles	Decreased acquisition	De La Fuente et al., 2007a; Kocan et al., 2009
Ubiquitin	Protein degradation	Decreased acquisition	De La Fuente et al., 2007a; Kocan et al., 2009
Glutathione S-transferase (GST)	Detoxification and signaling	Decreased acquisition	De La Fuente et al., 2007a; Kocan et al., 2009
Salivary selenoprotein M (SelM)	Protection against oxidative stress	Decreased acquisition	De La Fuente et al., 2007a; Kocan et al., 2009
α-1,3 fucosyltransferase (IsFT)	Glycosylation of proteins	Decreased acquisition	Pedra et al., 2010
G protein-coupled receptor Gβγ subunits (Gβγ)	Signal transduction	Decreased acquisition	Sultana et al., 2010
Phosphoinositide 3-kinase (pak1)	Cytoskeletal reorganization and signaling	Decreased acquisition	Sultana et al., 2010
p21-activated kinase (pi3k)	Cytoskeletal reorganization and signaling	Decreased acquisition	Sultana et al., 2010
Protein 11 (P11)	Unknown	Decreased acquisition	Liu et al., 2011
Heat-shock protein 20 (Hsp20)	Cellular stress response	Increased acquisition	Busby et al., 2012
Heat-shock protein 70 (Hsp70)	Cellular stress response	Decreased acquisition	Busby et al., 2012
Janus kinase (JAK)	Component of JAK/STAT signaling pathway	Increased acquisition	Liu et al., 2012
Signal transducer and activator of transcription (STAT)	Component of JAK/STAT signaling pathway	Increased acquisition and transmission	Liu et al., 2012
Gene-15	Antimicrobial peptide	Increased acquisition and transmission	Liu et al., 2012
X-linked inhibitor of apoptosis protein (XIAP)	E3 ubiquitin ligase	Increased acquisition	Severo et al., 2013
α-fodrin (CG8)	Spectrin α-chain	Decreased acquisition	Ayllón et al., 2013
Porin (T2)	Mitochondrial voltage-dependent anion-selective channel	Decreased acquisition	Ayllón et al., 2013

in an intracellular destruction and recycling of proteins in the proteasome, which is an important process also for the regulation of arthropod immune pathways (Ferrandon et al., 2007). Silencing of GST, vATPase or ubiquitin by RNAi decreases midgut *Anaplasma* acquisition in *D. variabilis* males fed on *A. marginale* infected cows, while silencing of GST or SelM decreases pathogen infection in the salivary glands of infected ticks fed on naïve sheep (De La Fuente et al., 2007a; Kocan et al., 2009). As previously mentioned, silencing of *D. variabilis* defensin named varisin that was shown to be expressed primarily in hemocytes, but also in midgut and other tissues (Hynes et al., 2008), decreased midgut pathogen acquisition in *D. variabilis* males fed on *A. marginale* infected cows and decreased infection in salivary glands of infected ticks fed on naïve sheep with obvious morphological abnormalities in bacterial colonies (Kocan et al., 2008b). Moreover, silencing of E3 ubiquitin ligase named x-linked inhibitor of apoptosis (XIAP) increases colonization of *I. scapularis* midgut cells and salivary glands by *A. phagocytophilum*, attracting even more attention to the ubiquitination process in ticks (Severo et al., 2013).

Fucosylation, which participates in many pathological processes in eukaryotes, has been shown to be modulated in ticks during *Anaplasma* infection (Pedra et al., 2010). *A. phagocytophilum* modulates expression of *I. scapularis* α-1,3 fucosyltransferase (IsFT) and uses α-1,3-fucosylation process to colonize the tick vector. Silencing of IsFT by RNAi reduces acquisition but not transmission of *A. phagocytophilum* in ticks.

The arthropod immune responses are generally regulated by Toll, Imd and JAK/STAT pathways (Ferrandon et al., 2007). Janus kinase (JAK)/signaling transducer activator of transcription (STAT) pathway has been shown to play a critical role in the tick defense against *Anaplasma* (Liu et al., 2012). Silencing of JAK/STAT genes by RNAi in *I. scapularis* causes burden of *A. phagocytophilum* in midgut, hemolymph and SG. The gene-15 of the salivary glands family encoding a member of 5.3-kDa antimicrobial peptide family is highly induced upon *Anaplasma* infection and regulated by JAK/STAT pathway. Silencing of gene-15 (and also STAT) by RNAi causes increased infection in salivary glands and transmission to the mammalian host.

Salivary protein 16 (Salp16) is an antigen recognized by tick-exposed host sera. Silencing of Salp16 by RNAi does not influence *A. phagocytophilum* acquisition in *I. scapularis* midgut, but the pathogen is not able to successfully infect the salivary glands (Sukumaran et al., 2006). Furthermore, expression of Salp16 in

the tick salivary glands is upregulated upon *Anaplasma* infection. It has been elegantly shown that Salp16 upregulation is not part of the tick defense mechanisms, but that *Anaplasma* selectively alter Salp16 expression for its benefit (Sultana et al., 2010). *A. phagocytophilum* infection induces actin phosphorylation, which is dependent on tick p21-activated kinase (ipak1)-mediated signaling. Activity of ipak1 is stimulated via G protein-coupled Gβγ receptor subunits (Gβγ), which in turn mediate phosphoinositide 3-kinase (pi3k) activation. In association with RNA polymerase II (RNAPII) and TATA box-binding protein, expression of Salp16 is selectively promoted. Silencing of ipak1, Gβγ or pi3k by RNAi reduces actin phosphorylation and *Anaplasma* acquisition by ticks (Sultana et al., 2010).

Recently, α-fodrin (spectrin α-chain) and mitochondrial porin (voltage-dependent anion-selective channel) were shown to be involved in *A. phagocytophilum* infection/multiplication and the tick cell response to infection in *I. scapularis* (Ayllón et al., 2013). The pathogen presence decreases expression of α-fodrin in the tick salivary glands and porin in both the midgut and salivary glands to inhibit apoptosis, subvert host cell defenses and increase infection. In the midgut, α-fodrin upregulation was used by the pathogen to increase infection due to cytoskeleton rearrangement that is required for pathogen infection. These results demonstrated that the pathogen uses similar strategies to establish infection in both vertebrate and invertebrate hosts.

After the initial infection of midgut cells, *Anaplasma* spread to other tick organs. However, the exact mechanism mediating migration to and infection of different tick organs is still not well known. Secreted *I. scapularis* protein 11 (P11), induced upon *A. phagocytophilum* infection, was shown to be important for *Anaplasma* migration from the midgut to the salivary glands, while being engulfed and hidden in the tick hemocytes (Liu et al., 2011). Silencing of P11 by RNAi or blocking the P11 with anti-sera or inhibition of hemocyte phagocytosis by injection of polystyrene beads into the tick hemolymph resulted in decreased *Anaplasma* infection of the tick salivary glands (Liu et al., 2011).

Tick subolesin (SUB), an ortholog of insect and vertebrate akirins, is possibly involved in several pathways, including innate immune responses, through a regulatory network involving cross-regulation between NF-κB (Relish) and SUB and SUB autoregulation (Naranjo et al., 2013). SUB is down-regulated during *A. phagocytophilum* infection of tick nymphs, but up-regulated in female midguts and salivary glands infected with *A. marginale* or *A. phagocytophilum* (De La Fuente et al., 2006; Galindo et al., 2009; Zivkovic et al., 2010; Merino et al., 2011; Busby et al., 2012). Silencing of SUB by RNAi has strong effect on tick mortality and feeding and causes degeneration of midgut, salivary glands and reproductive organs (De La Fuente et al., 2008). After SUB knockdown, infection with *A. marginale* is significantly reduced in *D. variabilis* male salivary glands, but has only little effect on infection with *A. phagocytophilum* (De La Fuente et al., 2006; Ayllón et al., 2013). Subolesin has been used for vaccination against tick infestations and pathogen infection (De La Fuente et al., 2011). Although limited success has been obtained in this area, ongoing efforts are focused on the characterization of the *Anaplasma*-tick interface to develop vaccines for the control of tick infestations and pathogen transmission (De La Fuente, 2012).

BABESIA

Babesiosis is a tick-borne malaria-like disease affecting health of many animals and reducing cattle production in tropical and subtropical regions worldwide. Moreover, human babesiosis increasingly raises public health concern (Florin-Christensen and Schnittger, 2009). *Babesia*, the causative agent of babesiosis, is an apicomplexan parasite, which is together with *Theileria* referred to as piroplasm because of its pear-shape intra-erythrocytic stage. The genus *Babesia* constitutes paraphyletic group of parasites (described in various hosts with discrepancies in developmental cycles), which can be only distinguished by an appropriate molecular methods (Allsopp and Allsopp, 2006). They multiply in vertebrate erythrocytes (asexual stage) and cause severe symptoms related to their destruction. In the tick (hard tick), the parasite undergo sexual development in the midgut content, multiply in midgut cells and spread to different tissues including the salivary glands and ovary. Most of *Babesia*, unlike *Theileria*, are capable of transovarial transmission and newly hatched larvae are infectious to the hosts (Chauvin et al., 2009; Florin-Christensen and Schnittger, 2009).

It has been shown that infection of ticks with *Babesia* parasite pose negative effect on the tick development (Cen-Aguilar et al., 1998), thus ticks are supposed to evolve defense mechanisms to control *Babesia* infection and to regulate their mutual interaction. Although genomic sequences of *Babesia* and tick are available (Pagel Van Zee et al., 2007; Cornillot et al., 2012) and several projects have identified tick genes differently expressed upon *Babesia* infection (Rachinsky et al., 2007, 2008; Antunes et al., 2012; Heekin et al., 2012), only few tick genes have been shown to be directly implicated in the vector-pathogen interaction (**Figure 4** and **Table 3**). First of them, called longicin (Tsuji and Fujisaki, 2007), is defensin-like protein of *H. longicornis* exerting anti-microbial and anti-fungal activity. Recombinant longicin was reported to inhibit proliferation of *Babesia* (*Theileria*) *equi* merozoites in *in vitro* cultures and to reduce parasitemia of mice experimentally infected with *B. microti*. Moreover, silencing of this gene by RNAi increased number of *B. gibsoni* in the tick midgut content, ovary and laid eggs, pointing to longicin role in the regulation of *H. longicornis* vectorial capacity (Tsuji et al., 2007).

Longipain (Tsuji et al., 2008) is midgut-specific cysteine protease of *H. longicornis*, whose expression is upregulated upon blood feeding. Similarly as for longicin, recombinant protein inhibited proliferation of *Babesia* (*Theileria*) *equi* merozoites in *in-vitro* cultures and silencing of this gene by RNAi resulted in increased number of parasites in the midgut lumen, ovary, and hatched larvae. In general, inhibition of tick and parasite proteases is of interest as both the tick and the parasite genomes encode for several cysteine proteases important for blood digestion (Sojka et al., 2008) and host invasion (Florin-Christensen and Schnittger, 2009), respectivelly. Addition of various cysteine protease inhibitors into the *B. bovis* culture resulted in parasite growth inhibition (Okubo et al., 2007). The cysteine proteases inhibitor called cystatin-2 (Hlcyst2) from *H. longicornis* (Zhou et al., 2006) was overexpressed in midgut and hemocytes after *Babesia* infection. Recombinant HlCyst2 had slight effect on *B. bovis* growth in *in vitro* assays, but

its role in the tick infection has never been experimentally examined.

Three tick genes, namely TROSPA, serum amyloid A and calreticulin has been recently identified by cDNA screen as genes upregulated after the tick infection with *B. bigemina* (Antunes et al., 2012). TROSPA is a midgut receptor with unknown function, which is used by *Borrelia* spirochete as a docking protein for midgut colonization and spirochete persistence (Pal et al., 2004). Serum amyloid A is a homolog of vertebrate acute phase protein reacting to inflammation (Urieli-Shoval et al., 2000). Calreticulin is an intracellular protein with many functions, including calcium binding, protein folding and immune signaling (Wang et al., 2012). Involvement of these genes in *Babesia* infection has been confirmed by RNAi, where silencing significantly reduced *B. bigemina* numbers in *Rhipicephalus annulatus* and *R. microplus* (Antunes et al., 2012). Furthermore, RNAi silencing of subolesin (the previously mentioned ortholog of mammalian akirin) or vaccination with recombinant SUB strongly reduced acquisition of *B. bigemina* by *R. microplus* fed on an infected cattle (Merino et al., 2011).

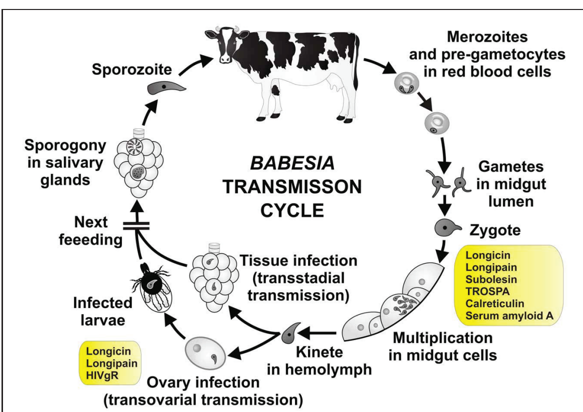

FIGURE 4 | Tick molecules involved in *Babesia* transmission. Schematic diagram representing general stages of *Babesia* infection and transmission (Zintl et al., 2003; Chauvin et al., 2009; Florin-Christensen and Schnittger, 2009). Tick genes, for which interference with *Babesia* acquisition and/or transmission have been proved by genetic tests, are shown in rectangles (see text and the **Table 3** for their function and references).
Pre-gametocytes in red blood cells, taken up within the blood meal, develop in the tick midgut content into matured gametocytes and gametes (ray bodies, Strahlenkörper) with distinctive spine-like projections. They fuse and give raise to the spherical spiked zygotes, which invade midgut cells. Inside the midgut cells, the zygotes transform, undergo meiosis and differentiate into motile prolonged kinetes (ookinetes). The kinetes escape the midgut cells, enter the hemolymph and invade other tick tissues, including ovary (transstadial and transovarial transmission, respectively). Here they undergo asexual reproduction and produce sporokinetes, which further spread the infection inside the tick or newly emerged larvae. During the next feeding, the kinetes that invaded salivary glands undergo a final cycle of multiplication (sporogony) to produce numerous sporozoites, host-invasive stages of the parasite. The sporozoites enter the tick saliva and infect host.

Vitellogenin serves as a storage protein and source of amino acids during embryogenesis and its uptake is achieved by a specific vitellogenin receptor, which was identified from *H. longicornis* and shown by RNAi to be indispensable for egg development (Boldbaatar et al., 2008). Interestingly, *Babesia* DNA was not detected in eggs lays from ticks with silenced vitellogenin receptor previously fed on dogs infected with *B. gibsoni*. This suggests that impairing the vitellogenin uptake interrupt the parasite transovarial transmission.

CONCLUSION—FUTURE PERSPECTIVES

The overall knowledge of tick innate immunity still lags far beyond the model invertebrate organisms and arthropod disease vectors. However, the availability of *I. scapularis* genome database (Megy et al., 2012), feasibility of functional genomics based on RNAi (De La Fuente et al., 2007b) and extensive number of tissue transcriptomes obtained from a variety of tick species promise to counterbalance experimental difficulties associated with tick handling and manipulation. Furthermore, introduction of the artificial membrane feeding (Krober and Guerin, 2007) extends our possibilities how to simulate the natural infections of ticks without the need of using laboratory animal models. These favorable conditions offer almost unlimited perspectives for the advanced research of the tick immune system and its impact on pathogen transmission. Among others, we can enumerate several high-priority topics, which can significantly aid to our understanding of the tick-pathogen relationship: (1) the role of epithelial immunity and maintenance of the redox balance for the pathogen persistence in the tick midgut; (2) interactions between the pathogens and commensal microflora; (3) tick antimicrobial peptides and their regulation via the Toll, Imd and JAK-STAT signaling pathways; (4) the role of tick primordial

Table 3 | Tick molecules interfering with *Babesia* acquisition and/or transmission.

Name	Supposed function	RNAi effect on the pathogen	References
Longicin	Defensin	Increased acquisition and transovarial transmission	Tsuji et al., 2007
Longipain	Cysteine protease	Increased acquisition and transovarial transmission	Tsuji et al., 2008
H. longicornis vitellogenin receptor (HlVgR)	Uptake of vitellogenin	Decreased transovarial transmission	Boldbaatar et al., 2008
Subolesin (SUB)	Component of the immune signaling pathways	Decreased acquisition	Merino et al., 2011
Tick receptor for OspA (TROSPA)	Unknown	Decreased acquisition	Antunes et al., 2012
Calreticulin	Protein folding and signaling	Decreased acquisition	Antunes et al., 2012
Serum amyloid A	Response to inflammation	Decreased acquisition	Antunes et al., 2012

complement system in the immune response against transmitted pathogens; (5) tick molecules involved in the pathogen acquisition, persistence or transmission as vaccine candidates; (6) the detailed description of the pathogen transmission cycles within the tick vector. Taken together, focused research in these areas can lead to the ultimate goal of efficient control of tick-borne diseases.

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Manuscript in preparation "Life cycle of piroplasms: comprehensive analysis"

The general consensus of piroplasms developmental cycle describes parasite asexual multiplication in the host blood cells and sexual development in the tick midgut followed by parasite invasion of internal tick tissues (e.g. [5, 13, 16, 65]). Nevertheless, the different species of the group *Piroplasmida* possess unique adaptations arising from their natural behavior, and thus significantly differ according to the vector species and reservoir hosts. Moreover, the phylogenetically distinct species vary in important characteristics of the transmission and multiplication in the host or vector cells. Therefore, the life cycle of piroplasms is considered as only partially elucidated due to inconsistency of clue events in parasite development. This is given by many partial descriptions spread in various publications, often published long time ago, and also due to recent changes in the nomenclature and redescription of many species (e.g. [21, 115, 116]). In the manuscript in preparation "Life cycle of piroplasms: comprehensive analysis" (pages 25-41) we made an effort to summarize all available knowledge of piroplasms development and make a uniform and comprehensive consensus of the problematics in context of recent phylogenetic studies [12].

Lifecycle of piroplasms: comprehensive analysis

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Piroplasms, *Babesia*, *Theileria*, developmental cycle, merogony, gamogony, sporogony

Abstract

The group *Piroplasmida* refers to intracellular tick-transmitted parasites with worldwide distribution. It comprises three genera – *Babesia*, *Theileria* and *Cytauxzoon* – posing significant risk to humans, livestock, wildlife and companion animals. Piroplasms are considered as one of the most common blood parasites distribute worldwide. The lifecycle of piroplasms is a series of complex events exploiting the blood cells of the mammalian host and internal tissues of the tick vector. Yet, the lifecycle of piroplasms is generally described very superficially since many inconsistencies occur. Such discrepancy is a result of only partial reports of crucial events in piroplasms lifecycle, nomenclature disunity and recent redescription of many species. This review intends to summarize the knowledge about piroplasms lifecycle with the respect of piroplasm evolution and provide a comprehensive view into the developmental events of *Babesia*, *Theileria* and *Cytauxzoon* parasites.

Pages 26-41 were removed because they contain unpublished data. If you are interested, please contact jalovecka@paru.cas.cz.

PART II. Summary of research methods

Part II. is summarizing methods used in the thesis with the exception of previously published papers [1, 117, 118] and manuscripts in preparation.

In this part, one methodological publication is included [117]:

Aase A, Hajdusek O, Øines Ø, Quarsten H, Wilhelmsson P, Herstad TK, Kjelland V, Sima R, Jalovecka M, Lindgren PE, Aaberge IS. Validate or falsify: Lessons learned from a microscopy method claimed to be useful for detecting *Borrelia* and *Babesia* organisms in human blood. Infect Dis. 2016;48(6):411-9.

Laboratory animals

We used 7 weeks old females of BALB/c laboratory mice (*Mus musculus*), supplied from Charles River Laboratories (VELAZ) or the Mongolian gerbil (*Meriones unguiculatus*; originally obtained from the same supplier). The guinea pigs (*Cavia porcellus*) were used to establish and maintain *Babesia*-free tick colony. Both gerbils and guinea pigs were bred in animal facility of the Institute of Parasitology, Biology Centre of CAS, Czech Republic. All experimental animals were treated in accordance with the Animal Protection Law of the Czech Republic No. 359/2012 Sb. and with the decree 419/2012 Sb. of Ministry of Agriculture on the protection of experimental animals (including relevant EU regulations).

Tick breeding colony

The *Babesia*-free colony of *I. ricinus* ticks was established by collection of adult ticks in landscape near Ceske Budejovice, Czech Republic by flagging method. The adult females were let to fully engorge on laboratory guinea pigs (*Cavia porcellus*) in the presence of tick males. The hatched larvae from laid eggs were tested for *Babesia* spp. infection (DNA extraction followed by PCR with *Babesia* spp. *18S* primers, Tab. 1) to exclude potential transovarial transmission of some *Babesia* species. Established tick colony was then reared in glass boxes with air humidity ~95% at 26°C constant temperature and photo-period 12h light and 12h dark at tick breeding facility of the Institute of Parasitology, Biology Centre of CAS, Czech Republic. Ticks in all stages of development (larvae, nymphs and adults) were used in the research; specification is included in each experiment description.

B. divergens in vitro cultivation

B. divergens parasite was isolated from bovine blood during the acute phases of babesiosis as described earlier [119] and cloned by limiting dilution. *B. divergens* was cultivated (37°C, 5% CO_2) in a suspension of bovine red blood cells (final concentration 1.5×10^9 cells/ml) in cultivation medium: RPMI 1640 medium containing L-glutamin and 25 mM Hepes (Lonza) with added gentamicin 50 µg/ml (Lonza), amphotericin B 0.25 µg/ml (Lonza) and 20% heat inactivated (56°C, 30 min) foetal calf serum (FCS, Lonza). Bovine red blood cells were obtained from a parasite-free cow (culture tested) by aseptic whole blood collection from indoor bred cows in a local slaughter house. Blood was manually defibrinated and 3 × washed in washing medium (cultivation medium without FCS) to remove buffy coat and serum, and stored at 4°C until use. In all experiments described in the thesis, the clone *B. divergens* 2210A G2 (origin Côtes d'Armor, France) was used, unless otherwise specified. Parasitemia of *in vitro* culture was monitored using erythrocytes smears stained by Diff-Quik Stain Set (Siemens); staining procedure was performed according to the manufacturer's instructions.

To cryopreserve the *in vitro* culture, infected red blood cells (parasitemia ~10%) were spun down (2000*g*, 5 min, 4°C) and the equivalent volume of FCS was added to pelleted cells. The mixture was then mixed with 30% glycerol (Sigma) in Alsever's Solution (Sigma) in ratio 2:1, respectively and after 20 min incubation at 4°C transported into liquid nitrogen. The de-freezing procedure consisted of melted sample washing by centrifugation (2000*g*, 5 min, 4°C) in washing medium; pelleted parasites were then added into the bovine erythrocytes suspension in cultivation medium (50 µl cells and 2.5 ml media).

B. microti in vivo maintenance

B. microti (Franca) Reichenow (PRA-99™), strain Peabody mjr. was obtained from ATCC: The Globar Bioresource Center (www.atcc.org). Parasite was maintained *in vivo* in laboratory BALB/c mice and passaged routinely every two weeks (the interval was set up based on *B. microti* growth curve detection, see Part IV.); the continuous long-term *in vitro* culture of *B. microti* has not been introduced yet. Passage of the infection into naïve mice was performed via intraperitoneal injection of infected blood (150µl per naïve mouse) obtained by cardiac puncture procedure of previously anesthetized infected mice. As an anesthetics, the mixture of 5% Narkamon (Spofa), 2% Rometar (Spofa) and 1× PBS (Phosphate Buffered Saline, pH 7.3) was used in ratio 8:2:10, respectively. Mice were anesthetized via intraperitoneal injection with the dose 100 µl of anesthetics per mouse. The infected blood was collected into citrate-phosphate-dextrose solution (Sigma Aldrich) in ratio 14:1, respectively to prevent coagulation. Parasitemia in mice was monitored on blood taken from the tail, smeared and stained by Diff-Quik Stain set; staining procedure followed manufacturer's instructions.

Parasites cryopreservation was performed by mixing of infected mouse blood and 30% glycerol (Sigma) in Alsever's Solution (Sigma) in ratio 2:1 followed by 20 min incubation in 4°C and final transport into the liquid nitrogen. The de-freezing procedure involved direct intraperitoneal injection of melted sample (~300 µl) into naïve laboratory mouse.

DNA extraction

DNA was extracted using NucleoSpin Tissue Kit (Macherey-Nagel) according to the manufacturer's instructions and stored at 4°C.

RNA extraction and cDNA preparation

Total RNA from whole tick bodies was extracted using NucleoSpin RNA II kit (Macherey-Nagel) according to the manufacturer's instructions. RNA concentration and quality was verified spectrometrically using NanoDrop (Thermo Scientific) and 1.5% agarose (Sigma) gel using Ethidium Bromide. Extracted RNA was reverse-transcribed into cDNA using Transcriptor High Fidelity cDNA

Pages 45-50 were removed because they contain unpublished data. If you are interested, please contact jalovecka@paru.cas.cz.

concentration 5 ng/ml in 2× SSC buffer) for 10 min at RT and again 2 × washed with 2× SSC buffer. Afterwards, samples were mounted onto glass slide using Fluoromount Aqueous Mounting Medium (Sigma) and examined by confocal microscope Olympus FW1000 under the magnification 100× - 6000×. Photos were processed in Fluoview software (FV10-ASW, version 1.7).

Transmission electron microscopy (TEM)

Dissected salivary glands (tick feeding time points are specified in description of Fig. 19) were fixed overnight in a mixture of 4% paraformaldehyde (Sigma) and 0.1% glutaraldehyde (Sigma) in 1× PBS pH 7.3 at 4°C. Subsequently, tissues were washed three times for 10 min in washing buffer (4% glucose (Sigma) in 1× PBS, pH 7.3) and dehydrated using ascending ethanol dilutions. Dehydrated samples were infiltrated in LR White resin (London Resin Company) in ratios of resin:95% ethanol 2:1, 1:1, 1:2 (1.5 h each, 4°C) and finally kept in pure LR White resin overnight at 4°C. Samples were then transferred to gelatin capsules (Polysciences) and filled with resin, which was allowed to polymerize for 24 h at 50°C. Samples were cut to ultrathin sections (90 nm), placed on copper grids, contrasted using ethanolic uranyl acetate for 30 min and lead citrate (Sigma) for 20 min. Tick salivary glands were observed in a JEOL 1010 transmission electron microscope.

Statistical analyses

Statistics was performed in R (version 3.4.0). At first, normality of dataset was tested with Kolmogorov-Smirnov test and the homogeneity of variances was evaluated using Bartlett test. If passed, the parametric statistical tests were used (t-test or ANOVA). If failed, the datasets were analyzed using nonparametric variants (Mann-Whitney test or Kruskal-Walis test, respectively). The tests are specified in description of each performed experiment (see Part III. and Part IV). The graphs were designed in GraphPad software (version 5).

Publication "Validate or falsify: Lessons learned from a microscopy method claimed to be useful for detecting *Borrelia* and *Babesia* organisms in human blood"

Human babesiosis is a disease with a growing incidence as apparent from the recently described clinical cases. Although the overwhelming majority of *Babesia* caused diseases are reported from United States [55], within last ten years the severe (and sometimes fatal) infections were documented in many of European countries; e.g. France and the British Isles [127], Czech Republic [82], Poland [128, 129], Finland [130], Sweden [131], Norway [132], Italy [67], Switzerland [133], Austria [67], Germany [68], Portugal [134] and Montenegro [135]. Nevertheless, the seroprevalence studies suggested that exists a large proportion of asymptomatic infections [127]. In general, babesiosis does not exhibit easily recognized clinical signs, and thus diagnostics is challenging [6, 13, 55, 70]. A definitive diagnosis is generally determined by microscopical examination of Giemsa-stained thin blood smears [56, 59, 65, 70] but at low parasitemia levels the parasite identification is laborious, with increased risk of parasite oversight [136, 137]. Moreover, the morphological appearance of *Babesia* intra-erythrocytic forms can be sometimes undistinguishable from the staining artefacts or could be misinterpreted as *Plasmodium* infection. The PCR represents a more sensitive identification tool but it strongly depends on the oligonucleotides used for parasite molecular detection; the method could face the problem to cover all potential parasite genospecies [138].

Therefore, we participated in the study driven by Department of Bacteriology and Immunology, Norwegian Institute of Public Health, Oslo, Norway in order to evaluate microscopy as diagnostics tool for *Babesia* (and *Borrelia*) and verify the microscopy results by reliable PCR method. The publication (pages 53-63) confirmed that microscopy could falsify the diagnosis because staining artefacts were often misinterpreted as *Babesia* infection. Thus, the definitive diagnosis of human babesiosis should include the molecular detection by PCR but rigorous precautions are required to avoid false-negative or false-positive results. I was involved in *Babesia* spp. detection in the human blood samples.

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ORIGINAL ARTICLE

Validate or falsify: Lessons learned from a microscopy method claimed to be useful for detecting *Borrelia* and *Babesia* organisms in human blood

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ABSTRACT

Background A modified microscopy protocol (the LM-method) was used to demonstrate what was interpreted as *Borrelia* spirochetes and later also *Babesia* sp., in peripheral blood from patients. The method gained much publicity, but was not validated prior to publication, which became the purpose of this study using appropriate scientific methodology, including a control group. **Methods** Blood from 21 patients previously interpreted as positive for *Borrelia* and/or *Babesia* infection by the LM-method and 41 healthy controls without known history of tick bite were collected, blinded and analysed for these pathogens by microscopy in two laboratories by the LM-method and conventional method, respectively, by PCR methods in five laboratories and by serology in one laboratory. **Results** Microscopy by the LM-method identified structures claimed to be *Borrelia*- and/or *Babesia* in 66% of the blood samples of the patient group and in 85% in the healthy control group. Microscopy by the conventional method for *Babesia* only did not identify *Babesia* in any samples. PCR analysis detected *Borrelia* DNA in one sample of the patient group and in eight samples of the control group; whereas *Babesia* DNA was not detected in any of the blood samples using molecular methods. **Conclusions** The structures interpreted as *Borrelia* and *Babesia* by the LM-method could not be verified by PCR. The method was, thus, falsified. This study underlines the importance of doing proper test validation before new or modified assays are introduced.

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Introduction

The diagnosis of various infections following tick bite may be challenging.[1–3] Lyme borreliosis (LB), also called Lyme disease, is caused by infection with the spirochete *Borrelia burgdorferi* sensu lato and remains the most prevalent tick-borne infection in Europe and Northern America. This bacterium has a very complex genome and may change phenotypical expression and biological function depending on natural environments (reviewed by Samuels and Radolf [4]) and may reveal different morphological variants, at least *in vitro*, although their role in LB could not be confirmed.[5]

Borrelia miyamotoi is another *Borrelia* sp. related to the relapsing fever *Borrelia* group and has recently been found in ticks in Norway.[6] Although fever seems to be a common clinical manifestation of *B. miyamotoi* infection, other non-specific symptoms may be present and, unlike *B. burgdorferi* s.l., *B. miyamotoi* is detected in blood of infected patients by PCR or by microscopy.[7,8]

Direct identification of the *B. burgdorferi* s.l. in blood by polymerase chain reaction (PCR) has relatively low diagnostic sensitivity, assumedly due to the low number of spirochetes in blood and temporary bacteremic phase,[2,9] whereas PCR on biopsy material or synovial fluid are more suitable for the detection of *Borrelia* spp. in specific disease manifestations. Accordingly, cultivation of *B. burgdorferi* s.l. from blood specimens has low sensitivity and, when present, they require special growth media and long incubation periods before they can be detected by microscopy. Hence, indirect detection methods such as identification of *Borrelia*-specific antibodies in serum or cerebrospinal fluid by ELISA or immune blot remain the most used methods in clinical diagnosis.[2] The sensitivity of the serological methods has been improved and the detection rate for disseminated early disease is 70–90% and for late disease (6–8 weeks after onset of symptoms) nearly 100%.[10–12] However, the interpretation of positive samples is hampered by a high seroprevalence of antibodies against *Borrelia* spp. in the general population, particularly in endemic areas.[13–15]

Babesiosis is another tick-borne disease that may affect humans, particular immunocompromised individuals.[16,17] Studies from Norway indicate, however, that human

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babesiosis is not a siginificant problem.[18,19] *Babesia* spp. are hemoprotozooan parasites that infect erythrocytes and microscopy of thin blood smears with Giemsa or Wright stains is a well-recognised method to identify *Babesia* parasites in blood from infected patients.[16,20] However, at low parasitemia the parasites may be difficult to identify and overlooked by microscopy, and more sensitive tools such as PCR assays should be considered.[21,22] The PCR assays, on the other hand, rely on specific hybridisation of primers and probes and may, thus, face a challenge to cover all potential zoonotic genospecies of the *Babesia* [17] and *Borrelia* microbes. Co-infection of both *Borrelia* and *Babesia* and other tick-transmitted pathogens may also occur.[23,24]

Some patients who suffer from long-standing disease attribute their symptoms to ongoing infections by these pathogens, although conventional laboratory methods have been negative for these diagnoses. In order to get a diagnosis and treatment regimens, they may seek alternative diagnostic methods. However, several of these alternative methods are not validated to be used for clinical diagnosis.

Microscopy has been used to identify *Borrelia* in different specimens from patients with suspected LB, particularly in specific biopsy material, e.g. skin biopsies.[2] In a recent study, microscopy of peripheral blood from patients by a modified method (the LM-method) was used to demonstrate what was interpreted as *Borrelia* spirochetes.[25] The method was later also used to detect what was assumed to be *Babesia* sp. in patients' blood (unpublished observations). The LM-method has gained much publicity in Norway and the patients and their organisations advocated for this test. However, the original report did not include any control group, no statistics and the structures observed were not verified to be *Borrelia* spirochetes by other technics. The purpose of this study was to confirm or falsify the reliability of the published LM microscopy method [25] as a test for *Borrelia* and *Babesia* infections. Blood samples from patients, previously judged positive for these micro-organisms by the LM-method and blood samples from healthy controls were analysed in two laboratories by microscopy either using the LM-method (looking for *Borrelia* and *Babesia*) or conventional method (looking for *Babesia* only), by PCR in five independent laboratories and by serology in one laboratory.

Materials and methods

Patients and controls

The patients recruited to participate in this study had previously been judged positive for *Borrelia* and/or *Babesia* with the microscopy method evaluated in this study. They came from different regions of Southern Norway and had initially heard about the modified microscopy method through media and patients organisations. They had made contact personally to the laboratory performing the modified method to have their blood analysed by this novel method.[25] Many of the patients attributed their symptoms to what is referred to as chronic LB. Twenty-one patients were included, with a median age of 39 years (range = 20–72 years) and 52% were male. Based upon a questionnaire, 76% had a known history of tick bite, whereas only 14% recalled to have tested positive for *Borrelia* with conventional tests like ELISA or immunoblot. Most had suffered or were still suffering from various neurological and muscular symptoms, like fatigue and muscular pain/weakness, for several years (median = 5 years, range = 1–26 years). Eighty-one per cent had received one or several antibiotic treatments for *Borrelia* infection according to the standard Norwegian regimen [3,26] or treatment for an extended period of time, some with relief of symptoms. Some patients also reported erythema migrans and some reported co-infections with other tick-transmitted pathogens.

The control group was recruited among healthy persons who did not recall to have had any tick-bite. Forty-one individuals (median age = 40 years, range = 21–64 years, 15% male) were included in the control group.

The study was approved by the Norwegian Regional Committees for Medical and Health Research Ethics (2013/2308) and all participants (both patients and controls) provided written informed consent to take part in the study.

Blood sampling and shipment to laboratories

The patients' blood was drawn by their general practitioners, collected in ethylenediaminetetraacetic acid (EDTA) tubes and sent to the laboratory at the Norwegian Institute of Public Health (NIPH). The samples were received within the same or the next day. EDTA blood from the controls was drawn by the staff at the laboratory at NIPH. In the laboratory at NIPH the blood samples were anonymised and given a randomised number, aliquoted into sterile polypropylene tubes and shipped to the analysing laboratories by ordinary postal service or by courier delivery at ambient temperature. The blood reached the laboratories regularly within the next 24 h.

In addition, control samples were sent to the laboratories to check results for consistency, i.e. blood from one of the control persons was collected at three different timepoints throughout the collection period. Also, one positive control sample for *Borrelia* and one for *Babesia* were prepared by adding *B. afzelii* strain ACA-1 (approximately five *Borrelia* spirochetes per 100 red blood cells), grown in BSK-II medium and *Babesia divergens* infected bovine blood (approximately five *Babesia* parasites per 100 red blood cells), respectively, to extra blood samples from another control person. These control samples were blinded and sent as normal samples to the laboratories.

Microscopy

Microscopy in Lab. 1 used the modified protocol, the LM-method, by the inventors of the method.

Borrelia

The modified protocol has briefly been described in a previous publication.[25] Six microlitres of blood was placed at the centre of the lower 1/3 of each microscope slide. A solution of 250 µl sodium citrate (0.65 g/100 ml distilled water) was added next to the blood droplet, touching the droplet and

left for 1 min. Next, a cover-slip (24 × 36 mm) was carefully put on so the blood-sodium-citrate mixture seeped in from the short edge between the cover-glass and the slide. The slides were put into an immune stain moisture chamber for 24–48 h at room temperature. It is claimed that this will allow the *Borrelia* spirochetes to replicate and enhance visualisation.[25] Then 1–2 drops of a solution of acridine-orange (1:10 000 in sodium citrate solution) was added to the edges of the cover-slide and incubated for 30 min before inspection. DNA containing nucleoids show as green fluorescent sites evenly scattered among RNA containing orange-red cytoplasm in the fluorescent microscope (450 nm excitation; barrier filter = 570 nm).

Babesia

Ten-to-twenty microlitres of blood was deposited at the lower part of a clean microscope slide. A blood smear was made by using another slide at 30° angel. For best staining, the blood smear dried at room temperature for at least 24 h, preferably 2 days, before continuing. The smears were fixed with 100% methanol for 2–3 min. Next, 1–2 ml Giemsa-solution was dripped onto the smear, after ~3 min 2 ml distilled water was added. The slides were agitated gently to mix the water with the colour and after 3 min the surplus colour was poured off. The slides were then washed several times with 96% ethanol. The preparations were left to air dry at room temperature for at least 15 min. For microscopy, 1–2 drops of immersion oil were dripped straight on the blood smear. The smears were then covered with a 0.17 mm thick cover glass. It then takes up to 48 h for the oil to penetrate the blood cells and the parasites to become clearly visible. The structures observed and interpreted to be different *Babesia* stages are often strongly stained and often expanded due to swelling. They occur in clusters among vast areas of non-infected cells.

A Zeiss Universal microscope equipped with 100 W halogene lamp, phase-contrast condensor 0.90/1.40, apochromatic phase optics, Zeiss Ph 63/1.40 oil immersion lens was used for microscopy. Digital cam: Logitech 5000pro or a Logitech 9000 web camera adapted to the microscope.

Microscopy in Lab. 2 used a conventional method, looking only for *Babesia*: Blood smears were stained with Diff-Quick Stain Set (Polysciences Europe GmbH, Eppelheim, Germany) and analysed for the presence of *Babesia* parasites using a conventional microscope (Olympus BX53F) and conventional morphological criteria.

Detection of Borrelia spp. and Babesia spp. by PCR

Lab. 2

DNA was extracted from 200 µl of human EDTA blood using commercial DNA isolation kit (Macherey-Nagel, elution to 100 µl). Quality of the DNA was checked by PCR amplification of human control genes.[27] Presence of *Borrelia* (*B. burgdorferi* s.l. and *B. miyamotoi*) or *Babesia* spp. DNA in the extracted samples was tested by PCR, in duplicate using two different volumes of DNA.[28,29] *Babesia* or *Borrelia* positive samples were sequenced or genotyped by PCR, respectively.[30]

Lab. 3

DNA from two different fractions of EDTA blood (1.5–5 ml) was isolated for each patient: (1) The blood sample was homogenised by inverting the tube 5–6 times and 200 µl blood was collected for extraction of total DNA by QIAamp DNA Mini Kit (Qiagen, Venlo, The Netherlands) as described by the manufacturer's instruction. (2) The remaining blood was centrifuged at 100 g for 15 min.[31] The plasma/buffy coat fraction was collected and centrifuged at 13 000 g for 60 min. All material from the pellet in 200 µl plasma was either further processed immediately or stored at −70 °C until processing. The pellet/plasma sample was incubated in ATL lysis buffer (Qiagen DNA Mini kit) supplemented with proteinase K for 1 h at 56 °C. Thereafter, the sample was processed as described by the manufacturer's instruction. The isolated DNA was stored at −20 °C for later analyses.

Two real-time protocols, targeting the ospA and 16S rDNA genes of *B. burgdorferi* s.l, were performed as previously described.[32] All samples were analysed once, except for one weak positive sample which was confirmed to be positive by a second run in triplicate with both PCR protocols on the same DNA isolate.

Lab. 4

DNA was extracted from a total of 100 µl EDTA blood using the semiautomatic Qiacube instrument (Qiagen, Duren, Germany). The tissue protocol for DNAeasy mini spin column, with off board lysis (1.5–3 h) using ATL lysis buffer and Proteinase K prior to loading onto the instrument. Samples were stored at −20 °C prior to analysis. Any samples rendering any signals/results were re-extracted and re-analysed to confirm positive/negative results.

Detection of *Borrelia* spp. was carried out by the multiplex realtime-PCR, slightly modified from Courtney et al. [33] Each assay was performed as a single reaction. Realtime amplification was performed using Brilliant III Ultra-Fast Probe QPCR mastermix (Agilent, Santa Clara, CA) and a total of 25 µl reaction volume (3 µl DNA) was run on Agilent Mx3005P QPCR Systems using a standard two step fast protocol (annealing temperature of 60 °C and 50 cycles) . Methods were previously tested on positive tick and DNA from animal blood.

Initial screening real-time PCR [34] for detection of *Babesia* was performed with Brilliant III Ultra-Fast Probe QPCR mastermix. Next, an in-house realtime PCR method targeting a different group of *Babesia* species was run (Øines, unpublished). Samples containing several non-*B. divergens*-like species of *Babesia*, including the zoonotic *B. microti*, remain negative using the standard screening realtime PCR,[34] due to differences in the DNA, so the additional realtime was used to pick up additional *Babesia* spp. which might be present.

Third, a nested PCR protocol [35] for amplification of various piroplasm DNA was used, to allow an even broader approach for detection and characterisation of a range of various piroplasms, if present in the samples. This method also allowed characterisation and identification using phylogenetic analysis of sequences from the positive spiked samples identified from the PCR analyses. This approach allowed

Table 1. Overview of assays performed (X) and laboratory.

	Microscopy LM-method	Microscopy conventional	PCR *Bb* s.l.*	PCR *B. miyamotoi*	PCR *Babesia* spp.	Anti-*Borrelia* spp. IgG and IgM
Lab. 1	X[a]	–	–	–	–	–
Lab. 2	–	X[b]	X	X	X	–
Lab. 3	–	–	X	–	–	–
Lab. 4	–	–	X	–	X	–
Lab. 5	–	–	X	X	–	–
Lab. 6	–	–	–	X	–	–
Lab. 7	–	–	–	–	–	X

*B. burgdorferi s.l.
[a]The method evaluated in this study.
[b]Only for Babesia spp.

correct species identification of the control, using the conditions previously reported.[36]

All analyses were performed in duplicate. The combination of methods was selected to enable an as broad as possible approach for identification of known *or* unknown species of *Babesia* or other related parasites, which may or may not have been present in any of the samples analysed.

Lab. 5

Total nucleic acid (NA) from 100 μl of blood was isolated using DNeasy® Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol, with the exception of not adding RNase A to the samples.

Reverse-transcriptase synthesis was performed using Illustra™ Ready-to-Go RT-PCR Beads Kit (GE Healthcare, Amersham Place, UK) according to the manufacturer's protocol and 10 μl (0.25 μg/μl) random hexamer primers (pd(N)6) were incubated with 20 μl isolated total NA for 5 min at 97 °C. Beads were dissolved in 20 μl RNAse free water and transferred to the solution containing total NA and primers, followed by incubation for 30 min at 42 °C and subsequently for 5 min at 95 °C, resulting in a final volume of 50 μl cDNA/DNA.

Four microlitres of cDNA/DNA per reaction were used in a Light Upon eXtension™ (LUX) real-time PCR assay to detect a 131 bp long fragment of the *Borrelia* 16S rRNA gene, using genus-specific primers, as previously described.[37]

Samples positive by the LUX real-time PCR assay were further analysed to identify species by sequencing the non-coding intergenic spacer regions 5S–23S and 16S–23S, as previously described.[37]

The method described above was originally developed for research purposes on ticks. Therefore, we evaluated the method on blood samples containing 100 μl human EDTA blood spiked with a known number of *Borrelia burgdorferi* sensu stricto B31 cells (ranging from 10^8 cells to 10^1 cells per sample). The method was able to detect the *Borrelia* 16S rRNA gene in all spiked blood samples.

Lab. 6

DNA extracted by Lab. 3 was delivered by courier to Lab. 6 and analysed for the presence of *B. miyamotoi* by a real-time PCR assay with probe and primers specific for a section of the 16S rRNA gene, as previously described.[6] The samples identified as *Borrelia* positive by Lab. 3 were further analysed by Lab. 6 to identify genospecies by direct sequencing of the chromosome located rrs (16S)-rrlA (23S) intergenic spacer (IGS), as previously described.[38]

Serology

The presence of antibodies against *Borrelia* spp. was analysed in Lab. 7 with recomBead (Mikrogen GMBH, Neuried, Germany) [39] and by Enzygnost® Lyme link VlsE/IgG and Enzygnost® Borreliosis/IgM (Siemens AG, Munich, Germany) according to manufacturer's instruction. The recomBead assay quantifies specific antibodies against a range of individual *B. burgdorferi* s.l. antigens and covering several genospecies (p100, VlsE, p58, p39, OspA, OspC *B.burgdorferi* sensu stricto, OspC *B. afzelii*, OspC *B. garinii*, p18B.ss., p18 *B. afzelii*, p18 *B. bavariensis*, p18 *B. garinii*, p18 *B. spielmanii*). The interpretation of positive and negative results are given according to diagnostic cut-offs described in the manufacturers' instruction.

Overview of the assays performed and the different laboratories is presented in Table 1.

Statistics

The proportion of responders and 95% confidence interval (CI) from proportion were calculated using GraphPad Prism, Ver. 6 and GraphPad Instat ver. 2.05 (La Jolla, CA).

Results

Microscopy

In Lab. 1 using the LM-method (modified protocol), both blood smears stained with Giemsa and blood incubated on the slide under the cover-glass were inspected regularly for the next days, using ordinary microscopy and phase contrast microscopy. Blood from 21 patients, 41 healthy controls and four control samples were analysed. Structures interpreted as *Babesia* were best seen after Giemsa staining, whereas structures interpreted as *Borrelia* became best visible after incubation for hours or days (Figure 1). The presence of these structures were given a score from negative to 4+, depending on how many such structures were observed. The results are presented in Figure 2. Using the LM-method, Lab. 1 observed structures interpreted as *Borrelia* in 11 patient samples (52%) and *Babesia* in 12 patient samples (57%) (Table 2). In nine patient samples

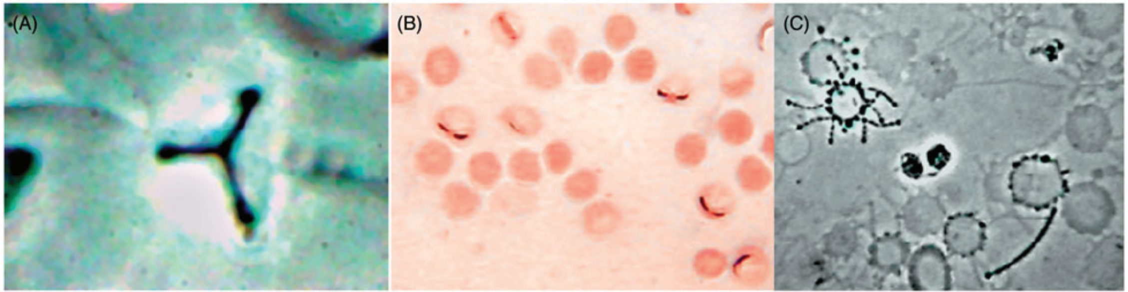

Figure 1. Photomicrograph by Lab. 1 using the modified microscopy method (LM-method), interpreted as: (a) a *Babesia*-like 'Maltese cross' in a sample from a healthy control subject (subject no. 8), (b) dividing *Babesia*, probably merozoite pairs, in a sample from a healthy control subject (no. 22, graded as 3+) and (c) a *Borrelia* pearl-chain structure and a nearly straight L-form (lower right) in a sample from a healthy control subject (no. 17, graded as 4+).

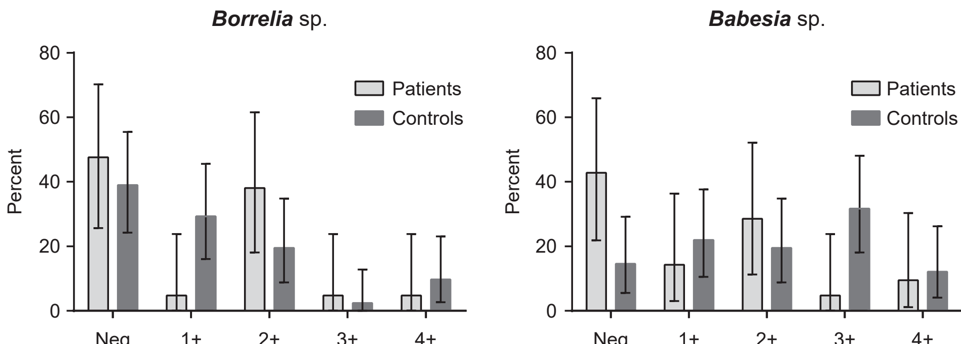

Figure 2. Microscopy results by the LM-method in Lab. 1. The bar graph showing the distribution of the microscope findings of *Borrelia*- (left) and *Babesia*- (right) like structures, graded from 0 (no *Borrelia*- or *Babesia*-like structure identified) to 4+. The error bars represent 95% confidence intervals from the proportion.

Table 2. Proportion (%) of subjects positive for *Borrelia* and *Babesia* like structures (95% CI) by microscopy; Lab. 1 using the LM-method and Lab. 2 using conventional protocol of the patient group (n = 21) and the control group (n = 41).

	Lab. 1 (LM-method)		Lab. 2 (Conventional method)	
	Patient group	Control group	Patient group	Control group
Borrelia	52 (30–74)	61 (45–76)	nd*	nd
Babesia	57 (34–78)	85 (71–94)	0	0
Double infection	43 (22–66)	59 (42–74)	na*	na

nd, not done at this Lab; na, not applicable.

(43%) structures interpreted as both *Borrelia* and *Babesia* were seen. In seven of the patient samples (33%) none of these figures were seen, however, in five of these other bacteria-like structures were observed.

In the blood samples from the 41 healthy controls, using the LM-method, Lab-1 found what was considered to be *Borrelia* in 25 samples (61%) and *Babesia* in 35 samples (85%); 24 samples (59%) were interpreted as positive for both *Borrelia*- and *Babesia* (Figure 1 and Table 2). Only six samples from the control group (15%) were negative for these structures, but they all contained other rod-like or cocci-like structures that were interpreted as bacteria. The results of the positive *Borrelia* and *Babesia* spiked blood samples and the repeated blood sampling are given in Table 3.

Lab. 2, which analysed for *Babesia* by the conventional microscopy protocol, did not find any *Babesia* sp. in any of the blood samples (Table 2).

PCR analysis

DNA analysis for the presence of *B. burgdorferi* s.l., *B. miyamotoi* and *Babesia* spp. was performed in different microbiological laboratories (Table 1). The methods employed in this study cover all the known human pathogenic *B. burgdorferi* s.l, *B. miyamotoi* and various *Babesia* which have been documented or are assumed to be present in Norway. Table 3 shows the individual results of the positive PCR results and the corresponding microscopy results.

All laboratories performing PCR analysis correctly identified the *Borrelia* and *Babesia* spiked positive control samples (Table 3).

PCR for Borrelia spp

Lab. 2 found *Borrelia*-specific DNA in five blood samples from the control group; three of these were weakly positive (1+) for what was interpreted as *Borrelia* by microscopy by the LM-method in Lab. 1, whereas two were negative by microscopy. Four of these were sequenced as *B. burgdorferi* sensu stricto and one was only specified as *Borrelia* sp. (Table 3).

Lab. 3 identified one sample with weak positive signals for *B. burgdorferi* s.l. in the control group; the isolated DNA was confirmed to be positive by a second run in triplicates with both PCR protocols and was sequenced to be *B. afzelii*. This sample was found negative for *Borrelia* (and *Babesia*) by microscopy by the LM-method and was also found to be negative at the other laboratories doing PCR.

Table 3. Corresponding microscopy findings of the PCR positive samples.

Group	PCR *Borrelia*				Microscopy *Borrelia*	PCR *Babesia*		Microscopy *Babesia*	
Patient/control	Lab. 2	Lab. 3	Lab. 4	Lab. 5	Lab. 1 (Laane)[a]	Lab. 2	Lab. 4	Lab. 1 (Laane)	Lab. 2 (Convent)
P	N	N	N	*B.* sp.	0	N	N	2	0
C	N	N	N	*B. afz.*	4	N	N	3	0
C	N	N	N	*B.* sp.	4	N	N	4	0
C	*Bb* s.s.	N	N	N	0	N	N	2	0
C	*Bb* s.l.	N	N	N	0	N	N	3	0
C	*Bb* s.s.	N	N	N	1	N	N	3	0
C	*Bb* s.s.	N	N	N	1	N	N	3	0
C	*Bb* s.s.	N	N	*B.* sp.	1	N	N	1	0
C	N	*B. afz.*	N	N	0	N	N	2	0
CtrA[b]	N	N	N	N	2	N	N	1	0
CtrA+*B.afz*	*B. afz*	*B. afz*	*Bb* s.l.	*B. afz*	0	N	N	0	0
CtrA+*B.div*[c]	N	nd	N	nd	2	*B.div*	*B.div*	3	0
CtrB 1[d]	N	N	N	N	4	N	N	4	0
CtrB 2	N	N	N	N	N	N	N	3	0
CtrB 3	N	N	N	N	2	N	N	3	0

N, negative; *Bb* s.l., *B. burgdorferi* sensu lato; Bb s.s., *B. burgdorferi* sensu stricto; *B. afz*, *B. afzelii*; nd, not done at this Lab.
[a]0–4 indicate an increasing number of *Borrelia*- and *Babesia*-like structures seen (0 = negative).
[b]CtrA is from a healthy person collected at different time points throughout the study period, CtrA were spiked with *B. afzelii* at one collection and *Babesia divergens* infected bovine blood at another collection.
[c]*B. divergens* infected bovine blood were added to blood from a healthy control (CtrA); this resulted in rapid agglutination and destruction of the bovine erythrocytes and most of the *Babesia* parasites.
[d]CtrB is from a healthy person (different from CtrA) collected at different time points throughout the study period (1: 17.06.14, 2: 03.09.14, 3: 16.12.14).

Lab. 4 found no PCR-positive *B. burgdorferi* s.l. in any of the samples from the patient or control groups.

Lab. 5 found one *Borrelia* PCR-positive sample in the patient group (no match was found upon sequencing); this sample was negative for *Borrelia* as interpreted by microscopy by the LM-method, and also negative by PCR in the other labs. In addition, Lab. 5 found three PCR-positive samples in the control group, one of these was sequenced as *B. afzelii*, one other was also positive by PCR in Lab. 2 and confirmed to be *B. burgdorferi* s.s. by sequencing in Lab. 2.

Labs. 2, 5 and 6 analysed the samples for the presence of *B. miyamotoi*, but no samples were found positive.

PCR for Babesia spp

Labs. 2 and 4, who looked for *Babesia* spp., did not detect *Babesia* spp. by PCR in any of the patient or control samples.

Serological results

Three of the patients' sera were scored as positive for IgG antibodies against *Borrelia* spp. by the Enzygnost assay. These were all negative by the PCR assays for *Borrelia* spp., whereas two were considered positive for *Borrelia* by microscopy by the LM-method. Four sera scored positive for IgM antibodies, both by the Enzygnost and the recomBead assays; these were also negative by the PCR assays, but three were found positive for *Borrelia* by microscopy. Blood from one healthy control had positive IgG test by the Enzygnost assay; this sample was found negative by both PCR and by microscopy for *Borrelia*.

In addition to interpret sera as positive or negative, the MicroBead assay also measures IgG and IgM antibodies against 13 single *B. burgdorferi* s.l. antigens, representing five different genospecies (listed in M&M). In the patient group, six of the 11 samples (55%) that were considered positive for *Borrelia* by microscopy were negative against all single antigens. Of the 25 blood samples in the control group that were interpreted as positive for *Borrelia* by the LM-method, 19 (76%) were negative for antibodies against all single antigens, whereas five revealed a weak binding to the VlsE or p100 antigens.

Discussion

To evaluate the modified microscopy method (the LM-method) for the detection of *Borrelia* and *Babesia* infections in humans, blood samples from 21 patients who attribute their symptoms to LB and 41 healthy controls were blinded and analysed for the presence of *Borrelia* spp. and *Babesia* spp. by microscopy using the LM-method, by a conventional microscopy protocol (*Babesia* only), by various PCR methods and for antibodies against *Borrelia* spp. By microscopy, using the LM-method (the method we wanted to evaluate in this study) Lab. 1 observed structures claimed to be *Borrelia*- and/or *Babesia* in 66% of the blood samples of the patient group and in 85% of the samples in the control group. The results suggest that this modified microscopy method does not distinguish between patients and healthy controls regarding infections with *Borrelia* spp. and *Babesia* spp.

The patient group is rather heterogeneous regarding symptoms, diagnosis, disease history and previous treatment. However, the patients included were all chosen among persons who previously had been judged positive for what was defined as *Borrelia* and/or *Babesia* by Lab. 1 using the LM-method. Only three of them had previously been tested positive for *Borrelia* spp. by conventional (serological) tests. Some have had symptoms for many years, others only for 1–2 years. However, this patient group is representative for patients who seek alternative diagnostic tests in their despair and struggle to get a diagnosis and effective treatment. Many of these patients ascribed their symptoms to what is referred to as chronic LB. The control group was adequately age-matched to the patient group, but it was under-represented

by males. However, the results do not indicate that gender is a contributing variable in these assays (data not shown).

The reported incidence of disseminated LB in Norway is ~4–6 per 100 000 annually (www.msis.no), whereas the incidence of localised disease (e.g. erythema migrans, which is not notifiable in Norway) is probably substantially higher. Seroprevalence studies show that up to 20% of healthy blood donors in endemic areas in Norway have antibodies against *B. burgdorferi* s.l., indicating exposure to *Borrelia* sp.[13,15] Regarding human babesiosis, only one autochthonous case has ever been described in Norway [18] and analysis of collected ticks identified *Babesia* spp. only in 0.9% of 1908 collected *I. ricinus* ticks;[36] thus, the real incidence of human babesiosis in Norway is not likely to be high. It should, however, be mentioned that the PCR method used in that study may have been biased against the *B. divergens*-like *Babesia* spp., as other zoonotic species like *B. microti* may not have been identified correctly using this method.

By microscopy using the LM-method, 38 blood samples were judged positive for what they interpreted as *Borrelia* structures; however, only five of these were positive for *Borrelia* spp. by PCR and positive PCR results were then only obtained in one, and for one sample in two, of the five PCR labs. No samples were positive for *Babesia* by the PCR methods used, thus the prevalent structures interpreted as *Borrelia*- and *Babesia* by the LM-method could not be verified as *Borrelia* or *Babesia* by the various PCR methods employed in this study. The rather inconsistent results from the PCR laboratories were somewhat surprising. Two laboratories identified *Borrelia* DNA in five (Lab. 2) and in one (Lab. 3) blood samples from the control group. The one blood sample identified by Lab. 3 and one of the samples identified by Lab. 2, was sequenced as *B. afzelii* and *B. burgdorferi* s.l., respectively; these were from two different healthy donors, but these samples were in the same shipment as the *B. afzelii*-spiked blood sample and, thus, contamination of samples may have occurred. The two other laboratories doing *Borrelia* PCR did not find these samples positive. It can, thus, not be excluded that there may have been some cross-contamination between the *Borrelia*-spiked samples and the other positive samples in Lab. 2 and Lab. 3. Furthermore, due to the low number of spirochete present in human samples, methods are designed to maximise sensitivity, for instance by targeting highly conserved 'household' genes and/or by applying a high number of PCR cycles. Although this may make the method more sensitive, the risk of acquiring false positive results is also increased. These points, as well as the fact that these samples were negative by serology, indicate that the positive results obtained rather reflect problems with specificity and/or cross-contamination, than reflecting true positive blood samples. The PCR laboratories were a mixture of clinical and research laboratories and our results envisage the need for improved quality control of the different in-house tests.

PCR on peripheral blood is not routinely used as a diagnostic test for LB due to its low diagnostic sensitivity.[9] Previous studies have shown PCR methods on peripheral blood may be highly sensitive, not only for hemoparasites like *Babesia* spp.,[21] but also for *Borrelia* spp. when the spirochetes are present in blood, as shown by *in vitro* studies by Chan et al. [40] The sensitivity of the PCR in our study could have been increased further by extracting DNA from an even larger blood volume. However, if 6–20 µl of blood is sufficient to see the microbes by microscopy, 100 µl to 5 ml of blood as used by the PCR labs should be plenty for DNA extraction and detection. It is well recognised that *Borrelia* spp. may adapt its genetic expression depending on the external environment. Whether such adaptations might delete all the targeted DNA sequences covered in our PCR assays is highly unlikely.

The presence of *Borrelia*-specific antibodies in some of the patients indicate previous exposure to the *Borrelia* spp., but cannot be interpreted as ongoing disease *per se*.[41]

The *Borrelia*- and *Babesia*-spiked samples from a healthy donor were all identified correctly by the PCR laboratories. Microscopy by the LM-method in Lab. 1 did not find *Borrelia* in the positive control blood, in spite of the high amount of *B. afzelii* added (about five *Borrelia* cells per 100 red blood cells). On the other hand, in a prior unspiked blood sample from the same donor collected 5 months earlier, a *Borrelia*-score of 2+ was observed using the same method, as was the same blood spiked with *Babesia* 1 week later. Similar variable results were observed for the consistency control sample. Along with this, the patient group was recruited among persons who were all judged positive for *Borrelia* and/or *Babesia* by the LM-method in Lab. 1 prior to this study, but, in the present study, 33% of the patients' samples were negative by microscopy for both these organisms using the same method. It may be argued that the patients have improved since last time and that the pathogens had disappeared from the blood.

Microscopy is regarded as the gold standard test for detecting *Babesia* in patients' blood. Pleomorphic structures, pyriform ring forms and Maltese crosses are easily seen in *Babesia*-infected erythrocytes on Giemsa stained blood smears. However, these parasites are not normally found in blood from healthy individuals.[21,22]

The *B. divergens*-spiked control was scored as 3+ by Lab. 1 using the LM-method, whereas Lab. 2 did not find any *Babesia* by conventional microscopy. It should be noted that the *Babesia* were cultivated in bovine blood, which was added directly to the human EDTA-blood collected from a healthy person. This resulted in massive agglutination of the bovine erythrocytes due to heterologous (anti-bovine erythrocytes) antibodies and, next, to lysis of the bovine blood. The *B. divergens*-spiked sample was, thus, not suitable for microscopy (whereas the DNA and, hence, the PCR assays is not affected). Control microscopy of the spiked blood the next day in the laboratory at NIPH revealed no remaining *Babesia*.

Some of the patients in this study are strongly affected by their symptoms and are desperate to find a diagnosis and treatment. Whether the observed structures considered to be *Borrelia* by Lab. 1 using the LM-method might be some non-pathogenic spirochete like *Treponema* sp. or other undefined microbes remains to be determined. However, incubation of blood in non-physiological conditions on the microscope slide may facilitate degradation and denaturation of cell structures that may give rise to 'pear-like structures', 'cystic forms', 'red blood cell blebbing products' and forms that may possibly

trap staining material like Giemsa and acridine orange and be misinterpreted as microbes.[42] Since these structures are even more prevalent and numerous in blood from healthy persons, it is unlikely that they are any pathogen or are linked to the patients' illnesses. However, given the range of PCR methods used in this study, and given the results across the groups, it seems unlikely that these structures are *Borrelia* spp. or *Babesia* spp.

Conclusion

Our results show that the structures claimed to be *Borrelia*- and *Babesia* by the LM microscopy method were equally found in healthy controls, they could not be verified by PCR. The microscopy method evaluated in this study can, thus, not be recommended to identify patients that should be treated for *Borrelia* or *Babesia* infections. This study underlines the importance of doing proper test validation before new or modified assays are introduced.[43]

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Disclosure statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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EDITORIAL COMMENTARY

Microscopy of human blood for *Borrelia burgdorferi* and *Babesia* without clinical or scientific rationale

In Norway, 'A simple method for the detection of live *Borrelia* spirochaetes in human blood using classical microscopy techniques' has attracted public discussion and media attention.[1] This method of 'simple microscopy' was developed by Mysterud and Laane from the department of Molecular Biosciences and Biology at Oslo University. A validation study has now been performed and published in the current issue of *Infectious Diseases*. The 'simple microscopy' was assessed thoroughly and found to be useless.[2] Masked samples from cases previously judged positive by the 'simple microscopy' and from a healthy control group were included. Using the 'simple microscopy', more than 50% of cases and more than 80% of controls were judged positive. PCR performed by independent laboratories did not detect *Borrelia* or *Babesia* DNA. *Babesia* was also not found by conventional microscopy.

In any case microscopy of blood is not a relevant procedure for the diagnosis of Lyme borreliosis for the following reason. Although haematogenous dissemination has been demonstrated by PCR and culture, the concentration of *B. burgdorferi* has been estimated to be about one bacterial cell in 10 ml of blood in patients with erythema migrans.[3,4] Thus, the concentration of *Borrelia* in patients has been shown to be too low to be found in a droplet of 6 µl on a microscope slide. A 10000-times higher concentration would be needed to achieve a reasonable sensitivity by microscopy.

'Simple microscopy' is not a relevant procedure for *Babesia* either, as conventional staining of the blood film is necessary for visualisation and correct identification.[5]

It would be unlikely that patients assumed to have concentrations of 1000 or more microorganisms per millilitre of blood have heterogeneous chronic symptoms for years without developing more severe pathology. By comparison, patients with high concentrations of microorganisms, as is usually seen with *Babesia* or relapsing fever *Borreliae*, are described as febrile and present with severe acute disease.[4,5]

The problem of misinterpretation and poor preparation of microscopic slides is historic. Koch,[6] in a publication from 1877, was very particular about methods for microscopy. Koch warns the reader of a swelling dirty stream ['... zu einem trüben Strohm anschwellen lassen...'] of publications on bacteriology. Koch stressed the importance of preserving the intact morphology of the microorganisms.

B. burgdorferi, in particular, has become the victim of a swelling dirty stream of problematic publications. In 2010 an independent review committee of the Infectious Diseases Society of America assessed 1025 publications and found a large volume of uncontrolled observations of doubtful scientific quality.[7,8]

The background for the study by Laane and Mysterud [1] is a speculative theory that morphological variants of microorganisms play a role in otherwise unexplained persisting symptoms. The literature does not support any causal relationship.[9]

Light microscopy is an important basic procedure in microbiology using wet mounts and stained preparations. How to perform microscopy is in the basic curriculum for undergraduate students in microbiology and for laboratory technicians. It is part of basic training to identify microorganisms and to distinguish tissue structures or artefacts.

It is reprehensible that pseudoscientific diagnostic testing was performed on human specimens at Oslo University. This practice has now been stopped, to my knowledge. However, non-validated diagnostic procedures are still be provided by alternative private laboratories and clinics and this has been warned against previously.[10–12]

If patients do not suffer from Lyme borreliosis or another tick-borne infection, then what is the problem?

Experience from a Dutch infectious disease clinic specialising in suspected Lyme borreliosis found 18 (9%) of 200 consecutive cases to have definite or probable *Borrelia* infection.[13] Notably, no alternative diagnosis was found in 77 (39%), despite a multidisciplinary approach. Probably most of the patients seeking alternative medical attention for a perceived tick-borne disease are recruited from the latter group without an 'alternative diagnosis'. This labelling illustrates the problem, as the patient may feel rejected and lose trust. The patients most likely suffer from a somatoform disorder and require psychological and social support.[14] The psychological attention should start concurrent with the standard clinical work-up, not at a later stage, in order stop to progression of somatoform disorders. It is also recommended to avoid unnecessary diagnostic testing.[15] *Borrelia* serology should be avoided in patients with diffuse symptoms without objective designs of disease.[16] The positive predictive values will be low (<1%) and a positive serology result will only contribute to unnecessary concern.

In conclusion, Aase et al. [2] should be recommended for their effort to falsify inappropriate microscopy. The study shows the fundamental importance of a control group for evaluation of diagnostic accuracy and that cases with disease should be selected according to appropriate case definitions. I hope the study serves as a warning against non-validated microscopic procedures and helps prevent mismanagement of patients with chronic complaints, who are lured to seek improper diagnosis in the future.

Disclosure statement

The author reports no conflicts of interest. The author alone is responsible for the content and writing of the paper.

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Appendix to publication "Validate or falsify: Lessons learned from a microscopy method claimed to be useful for detecting *Borrelia* and *Babesia* organisms in human blood"

For the study, we used nested PCR method based on identification of *Babesia* spp. *18s* gene [116]. The sensitivity of technique was evaluated using *B. microti* infected mouse blood (Fig. 5). The detection limit was determined of 12 infected erythrocytes in the PCR reaction (3×10^{-5} % parasitemia). Moreover, the method detects wide spectrum of *Babesia* species and following sequencing of PCR amplicon allows parasite species identification; we successfully distinguished the most common agents of human babesiosis, *B. divergens, B. venatorum* and *B. microti*.

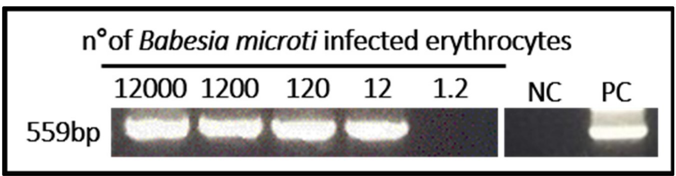

Figure 5. The sensitivity of the technique used for routine *Babesia* spp. detection. The mouse blood with parasitemia 0.03% was proceeded 10fold series dilution (in uninfected mouse blood) and extracted DNA was analyzed by PCR using the primers for *18S* gene. NC = negative control. PC = positive control.

PART III. Establishment of *Babesia divergens* laboratory model and its experimental application

Part III. is summarizing experiments performed in order to introduce complete laboratory model of *B. divergens.* Although we did not establish the complete *B. divergens* laboratory cycle, we report here the conducted experiments (unpublished) and include one publication elucidating the sexual commitment of *B. divergens*. Moreover, the optimized techniques enabled us to follow a new direction of *B. divergens* research, described in the manuscript in preparation "Validation of *Babesia* proteasome as a drug target".

In this part, two research publications are included, one published [118] and one in preparation:

Jalovecka M, Bonsergent C, Hajdusek O, Kopacek P, Malandrin L. Stimulation and quantification of *Babesia divergens* gametocytogenesis. Parasit Vectors. 2016;9(1):439. doi: 10.1186/s13071-016-1731-y. PubMed PMID: 27502772; PubMed Central PMCID: PMCPMC4977898.

Jalovecka M, Hartmann D, O'Donoghue AJ, Kopacek P, Hajdusek O., Sojka D. Validation of *Babesia* proteasome as a drug target. *In preparation*.

Babesia divergens: introduction to the problematics

Based on the most recent phylogenetic analyses, *B. divergens* belongs to the *Babesia sensu stricto* clade/group which refers to the monophyletic group characterized of transovarial transmission in ticks and multiplication exclusively in erythrocytes of the vertebrate host [5, 12, 18-20]. Recently, *B. divergens* was sequenced with no significant differences in size or structure from the other species of *Babesia sensu stricto* group, *B. bovis* and *B. bigemina* [139-141]; the genome size varied from ~9 to ~11.5 Mbp depending on the *B. divergens* strains [139, 140].

The life cycle as well as ultrastructure of *B. divergens* is nearly identical to other species from the *Babesia sensu stricto* group [12]. The natural transmission cycle (Fig. 6) comprises cattle as the mammalian host and *Ixodes* ticks as vectors. Actually, the cattle is considered as the reservoir host for *B. divergens* [57] where the asexual reproduction occurs. Among asexual stages, the first sexual stages – gametocytes – occur in the host erythrocytes [118, 142, 143] and further develop in the tick gut lumen into gametes, referred as ray bodies [54, 143]. Gametes then fuse and give rise to a zygote which is differentiating into kinetes [54]. For *B. divergens*, no postzygotic meiosis was described but it is assumed that *B. divergens* undergoes postzygotic development as other species from the group *Babesia sensu stricto* [53]. Kinetes migrate to the haemolymph and are spread through the tick body invading tick internal organs; for nymphal tick the invasion of salivary glands and following sporogony was documented [54]. The transovarial transmission of *B. divergens* was previously reported [144-147]. Therefore, the parasite kinetes had to invaded the ovary of adult females and fully matured sporozoites likely developed since the resulting larvae were infectious to the host [144].

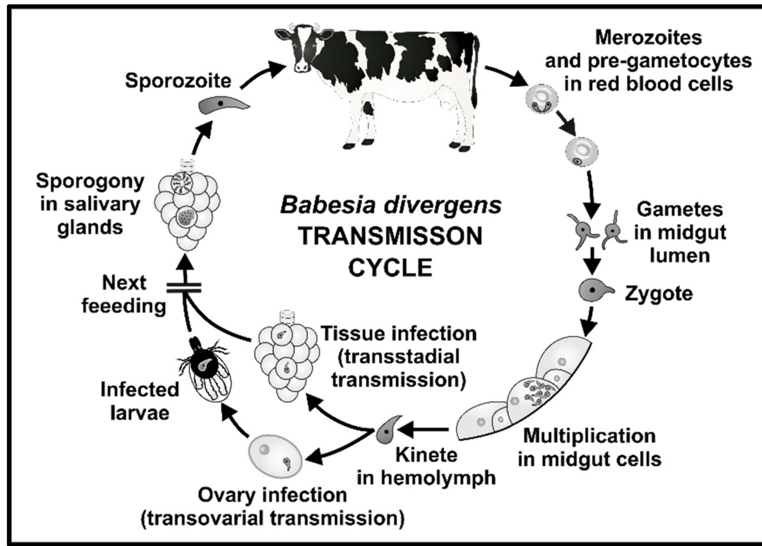

Figure 6. *B. divergens* transmission cycle. Natural transmission cycle of *B. divergens* involves cattle as the reservoir hosts and *I. ricinus* ticks as vectors. Parasites in bovine red blood cells are taken-up within the blood meal of adult tick female, where they undergo sexual reproduction and invade tick internal tissues of tick including ovary leading to the transovarial transmission into the larvae. Then parasite transmit transstadially to nymphs and adults. Adult female then usually spread the infection into the naïve cattle during the engorgement. Adapted from [1].

As other *Babesia* species, *B. divergens* in host erythrocytes undergoes asynchronous multiplication resulting in the presence of various asexual stages in bloodstream at the same time [57, 65]. The trophozoites are round or oval, commonly called the ring stages. The trophozoites morph into merozoites of typical piriform shape. Merozoites occur in clusters of two or four; the four merozoites in one cell are constituted into form of tetrads named Maltese cross [148]. The formation of Maltese cross is unique to *Babesia* and serves as distinguishing feature. The size and position of trophozoites and merozoites are usually dependent on the host species and *in vivo* or *in vitro* maintenance [57, 149-152]. To date, ultrastructural studies of *B. divergens* were performed just for stage of merozoites [153, 154] and no significant differences were observed in comparison with other species of the *Babesia sensu stricto* group [38, 155, 156].

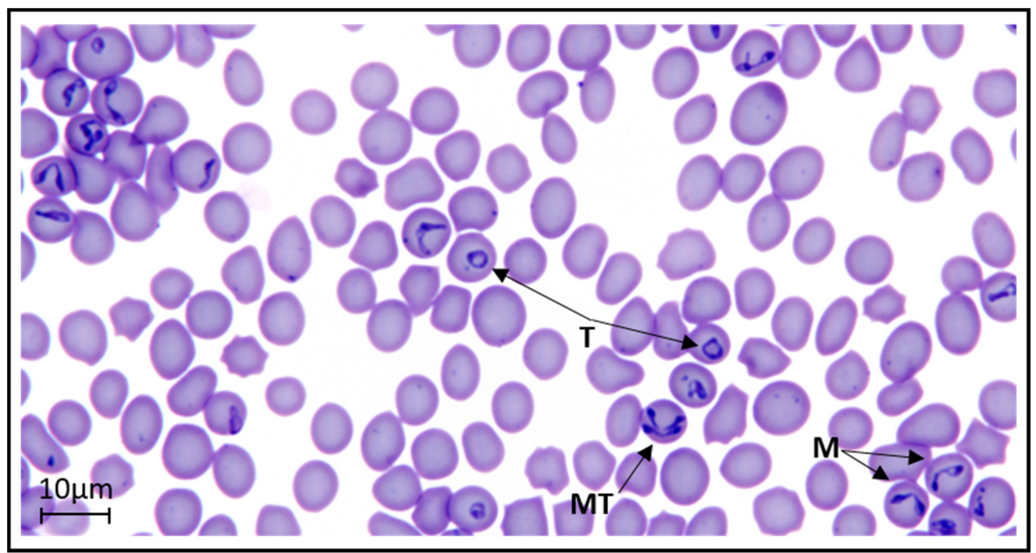

Figure 7. *B. divergens* in bovine erythrocytes cultivated *in vitro*. Smear was stained using DiffQuik staining set. M = merozoites. MT = merozoites clustered into a tetrad. T = trophozoites.

Currently, epidemiological surveys have documented the presence of *B. divergens* throughout various European countries, determined by the wide distribution of the tick vector, *I. ricinus* [4, 16, 57, 62, 157, 158]. All three life tick stages appear to be a competent vector of *B. divergens* [57, 157]. Based on feeding preferences, the larvae show limited importance in the babesiosis epidemiology contrary to nymphs and adults; particularly adult stages are believed to acquire infection [159]. *B. divergens* is maintained in the environment particularly by cattle previously recovered from disease or carrying subclinical infection [57]. The incidence of *B. divergens* in tick's vectors increases notably in areas with cattle breeding in Europe (L. Malandrin, oral communication). The cattle represent the reservoir host of *B. divergens*. Bovine babesiosis caused by *B. divergens* is considered as underestimated and economically important threat for current livestock industry in Europe [4, 57,

62] and causes severe epidemics in cattle, where the mortality of untreated cases may reach high numbers [57].

Compared to other bovine *Babesia* species (placed in *Babesia sensu stricto* group [12]), *B. divergens* infection was reported in a broader spectrum of hosts. Disease was manifested in splenectomised primates [160], splenectomised mouflon and various deer species [161], and splenectomised sheep [162] as well as in intact reindeer [163] and sheep [164]. However, recent description of *B. venatorum* (also reported as *Babesia* sp. EU1) is raising question about the correctness of previous studies. *B. venatorum* differs from *B. divergens* by host specificity (among others). Current knowledge attribute cervids (*Cervidae*) as the reservoir hosts of only *B. venatorum* [88-92], and cattle (*Bos taurus*) are believed to be the only "true" host of *B. divergens* [164].

B. divergens belongs to the species easily cultivated *in vitro* [119, 165] where it exhibits low host specificity to blood cells. The maintenance of the parasite was successfully adapted in bovine [119, 165], human [153, 166], rat [167], horse [150] and sheep [150, 162] erythrocytes. Moreover, a number of experiments were attempted in order to establish *B. divergens* in the laboratory animals. Common laboratory hosts like rabbits [160], mice, hamsters or rats [168, 169] were not susceptible to the infection, although the infection can fulminate in splenectomised rats [152]. The only laboratory animal found to be fully susceptible (whether splenectomised or intact) is the Mongolian gerbil (*Meriones unguiculatus*) [170] but the completely controlled transmission model *Babesia* - tick - gerbil has not yet been introduced. Yet, the transmission of *B. divergens* from experimentally infected gerbil to the tick *I. ricinus* was confirmed [54] and another study reported the infection of splenectomised gerbils by *I. ricinus* larvae, hatched from eggs laid by adult females previously fed on the infected calf [144].

In order to establish the transmission model of *B. divergens*, we conducted several experiments to reach the parasite acquisition by *I. ricinus* ticks. Here we summarize the obtained data and explain why the complete *B. divergens* laboratory cycle could not be introduced.

B. divergens infection of Mongolian gerbils and tick feeding

An evitable issue of transmission model establishment was to select the suitable and accessible laboratory host for *B. divergens* because cattle as an experimental animal possess administrative complication. Since the Mongolian gerbil (*Meriones unguiculatus*) has been found to be a fully susceptible laboratory animal to *B. divergens* infection [170], we performed several attempts to establish the infection of *B. divergens* in gerbils and standardize the course of infection. Gerbils kept in laboratory conditions were intraperitoneally (i.p.) infected with 150 µl dose of *B. divergens in vitro* culture at a 10% parasitemia per animal. Although we managed successful gerbil infection, we observed considerable differences in growth curves of *B. divergens* in gerbil blood stream and maximum parasitemia appeared. It was previously reported that many factors (e.g. parasite dose or gerbil age) could influence the development of the *B. divergens* infection [57, 144, 151, 170]. Thus, we used animals of different age and sex but no infection standardization was reached (Fig. 8). We observed that some experimental animals possessed very high parasitemia within one week after infection but it was necessary to euthanize the animals regarding their health condition. In addition, about one third of experimental animals appeared to be resistant, no parasite multiplication occurred in their bloodstream. The potential explanation of such results can be attributed to the genetic heterogeneity of the experimental gerbils despite the assumed "bottleneck" of animals bred in laboratory conditions.

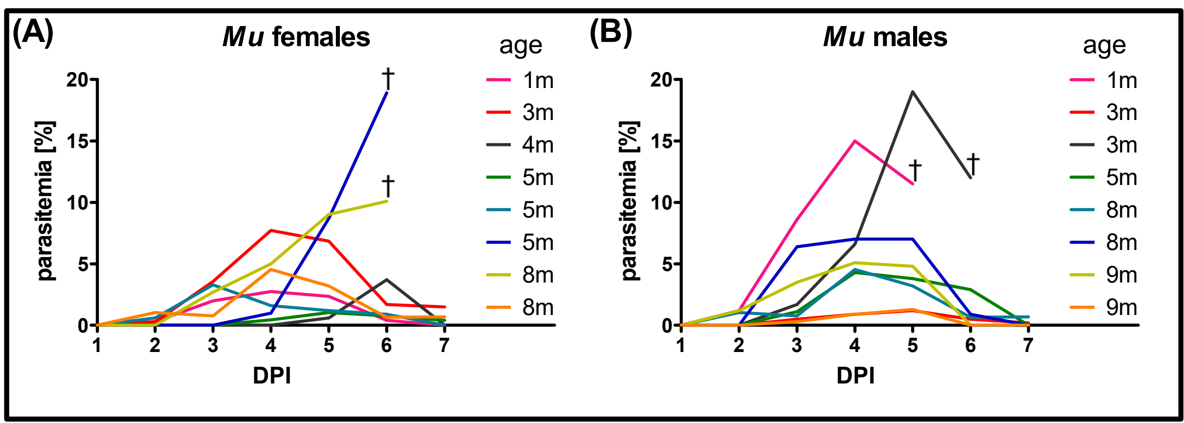

Figure 8. *B. divergens* infection of Mongolian gerbils (*Meriones unguiculatus*). Gerbil females (A) and males (B) were intraperitoneally infected with the same dose of parasites (150µl of *in vitro* culture with 10% parasitemia per animal). The course of infection was monitored daily from 1 DPI up to 7 DPI. Smears were stained using DiffQuik staining set, parasitemia was counted at 1000 RBCs. The legend reflects the age of the experimental animals (1m = 1 month). *Mu* = *Meriones unguiculatus.* = euthanasia † of experimental animals. DPI = days post infection. RBCs = red blood cells.

Despite these complications, we carried out numerous attempts to infect nymphal ticks. Although we obtained fully engorged nymphs, the infection was not transstadially transmitted to adults. To date, the acquisition of *B. divergens* by nymphal ticks fed on gerbils was confirmed only by DNA measurements [54]. The study described the parasite sexual development followed by kinetes

invasion to salivary glands but no transstadial transmission. The potential reason of such phenomenon can be attributed to incompetence of nymphal ticks to acquire and transmit the parasite. It is believed that natural transmission of *B. divergens* occurs via the feeding of adult stages of ticks on infected host and subsequent transovarial transmission to larvae. The successful *B. divergens* transmission via larval progeny was previously reported using adult *I. ricinus* females fed on infected splenectomised calves [144, 146, 147] but only one study documented the transmission of *B. divergens* to the gerbil via larvae feeding [144]. We attempted several times to feed adult *I. ricinus* females on infected gerbil but we could not reach fully engorgement; the discrepancy of *B. divergens* infectivity and multiplication still persisted. Based on observed complications, we decided to use artificial feeding [126] of tick females directly on *in vitro* culture of *B. divergens* in bovine erythrocytes.

Implementation of *in vitro* feeding technique for tick infection with *B. divergens*

Artificial feeding technique takes advantages of live animal replacement by *in vitro* culture using either a silicone membrane [126] or the skin of rabbit or gerbil [145, 171]. Tick artificial feeding had been previously used for a successful acquisition of *B. divergens* parasitized bovine erythrocytes cultivated *in vitro* into the *I. ricinus* tick females but the transmission cycle was not completed; feeding of infected larvae on gerbils did not resulted in host infection [145]. Since the *in vitro* feeding of ticks according Kröber and Guerin [126] was already implemented in our laboratory by my colleague (J. Perner, Institute of Parasitology, Biology Centre of CAS, Czech Republic; Fig. 9A), we adapted the technique for artificial infection of adult *I. ricinus* females on *B. divergens* infected erythrocytes.

Adult female ticks (bred in laboratory conditions) were fed on *B. divergens in vitro* culture of bovine erythrocytes in cultivation medium (specified in the Part II.). Unfortunately, in this case the ticks were not able to fully engorge (Fig. 9B). Subsequently we replaced the culture by full bovine blood enriched with *B. divergens* infected red blood cells (obtained from *in vitro* culture). We received fully engorged tick females (Fig. 9C) and in their organs we assessed the presence of *B. divergens* DNA using nested PCR with primers for *18S* gene (Tab. 1). At first, we did not detect any parasite DNA in tick organs except midgut (data not shown); the positive parasite detection in midgut is given by acquisition of intra-erythrocytic asexual stages by the blood meal. The negative detection of parasite DNA in tick ovaries and salivary glands implied the incompetence of parasite to developed sexually and further invade the tick internal tissues. The parasite sexual reproduction in the tick midgut is governed by gametocytes, intra-erythrocytic sexual stages imperative for tick infection [3, 39, 46, 172]. We hypothesized that parasite long-term *in vitro* maintenance diminished the parasite competence to produce gametocytes, as it was previously reported for *Plasmodium* [173].

Therefore, we verified the gametocytes production in the *in vitro* cultured parasite using the previously described and validated molecular markers for *B. divergens* gametocytes [142, 143]. The analysis confirmed that the parasite lost the ability of sexual commitment during the long term *in vitro* cultivation [118], and thus it cannot infect the tick.

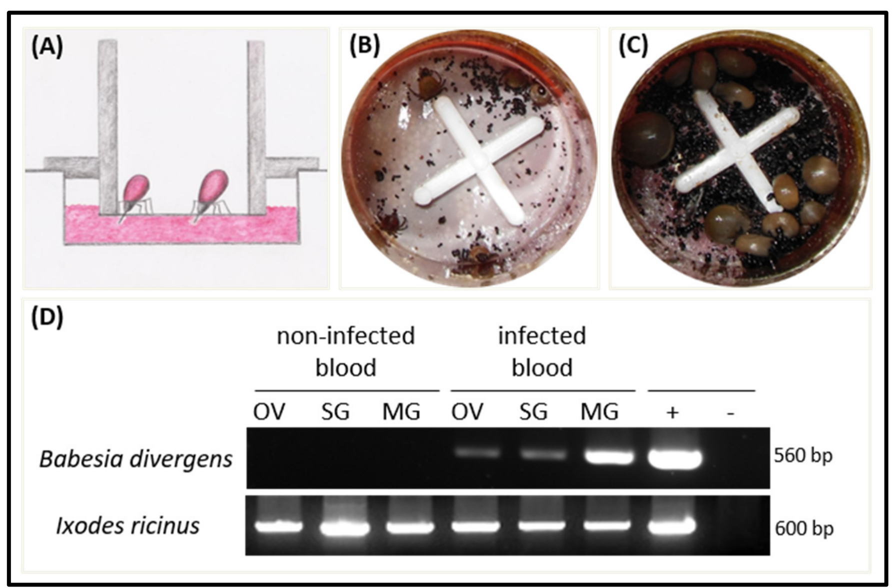

Figure 9. Implementation of *in vitro* feeding technique for tick infection with *B. divergens*. (A) Illustration of feeding unit with silicone membrane. *I. ricinus* females feeding on *B. divergens in vitro* culture (B) and on infected full bovine blood (C). Photos were taken on 7th day of continuous tick feeding on culture or blood with 10% parasitemia. *In vitro* culture or infected blood were changed each 8 hours. (D) *B. divergens* DNA detection in internal organs of fully engorged ticks artificially fed on either healthy or infected bovine blood including detection of *I. ricinus* DNA as control. OV = ovaria. SG = salivary glands. MG = midgut.

With respect to this result, we conducted the artificial feeding of tick females using the *B. divergens* strain with verified gametocytes production. We confirmed parasites DNA in all examined organs (ovaria, salivary glands and midgut) in ticks fed on infected blood (Fig. 9D) implying the successful tick infection. Yet, the contamination of tissues by infected blood meal during the tick dissection must be admitted. Unfortunately, the ticks kept for larvae hatching did not survived. Tick females laid only few eggs and no larvae were hatched. The obtained eggs did not contain parasite DNA but it was previously suggested that the first eggs in the clutch are usually parasite-free due to the length of sexual development of parasite in the tick internal tissues and the progress of eggs development (L. Malandrin, oral communication). We hypothesized that the lowering of the parasitemia could help since the high mortality may represent a sign of tick female infection because ticks fed on no-parasitized blood laid the standard amounts of eggs. In spite of several attempts to lower the parasite load in the tick blood meal, ticks still died prior or shortly after beginning of eggs laying. Thus, we had to conclude that establishment of *B. divergens* transmission cycle in the laboratory conditions represents very difficult task. This fact is supported by the lack of studies,

although the *B. divergens* maintenance in *in vitro* culture is feasible [119, 174]. Moreover, the authors of the only study reporting the transstadial and transovarial transmission of *B. divergens* by *I. ricinus* artificially fed ticks did not documented the living parasite stages and never succeeded to finalize the cycle by the host infection [145] .

Based on the previously described results, we decided to switch the *Babesia* species to *B. microti* in order to follow the aim of my doctoral thesis – the establishment of *Babesia* laboratory cycle to study a mutual immune interaction between the parasite and the tick vector. The results are thoroughly described and discussed in Part IV.

Publication "Stimulation and quantification of *Babesia divergens* gametocytogenesis"

The publication (pages 74-90) is focused on *B. divergens* sexual commitment which is still poorly understood part of parasite developmental cycle. During the optimization procedure in order to establish *B. divergens* laboratory cycle we realized the great significance of *B. divergens* sexual stages – gametocytes – an essential prerequisite to infect ticks. Taking advantage of previously described and validated molecular markers for *B. divergens* gametocytes [142, 143] – first sexual stages occurring in the blood stream of vertebrate host – we established a technique for the quantification of *B. divergens* gametocytes in cultures *in vitro.* Using this technique, we documented that parasite competence to of sexual commitment gradually decrease during the long-term cultivation and can be lost after several years of *in vitro* maintenance. We also monitored the kinetics of parasite sexual commitment upon the various stimuli in order to identify incentives significantly increasing the gametocytemia, and thus increase the parasite transmission efficiency. The research provided insight into *B. divergens* sexual development and has the potential to facilitate further research in the field of *Babesia*-tick interactions.

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Parasites & Vectors

RESEARCH Open Access

Stimulation and quantification of *Babesia divergens* gametocytogenesis

Marie Jalovecka[1,2,3,4*], Claire Bonsergent[1,2], Ondrej Hajdusek[3], Petr Kopacek[3] and Laurence Malandrin[1,2]

Abstract

Background: *Babesia divergens* is the most common blood parasite in Europe causing babesiosis, a tick-borne malaria-like disease. Despite an increasing focus on *B. divergens*, especially regarding veterinary and human medicine, the sexual development of *Babesia* is poorly understood. Development of *Babesia* sexual stages in the host blood (gametocytes) plays a decisive role in parasite acquisition by the tick vector. However, the exact mechanism of gametocytogenesis is still unexplained.

Methods: *Babesia divergens* gametocytes are characterized by expression of *bdccp1*, *bdccp2* and *bdccp3* genes. Using previously described sequences of *bdccp1*, *bdccp2* and *bdccp3*, we have established a quantitative real-time PCR (qRT-PCR) assay for detection and assessment of the efficiency of *B. divergens* gametocytes production in bovine blood. We analysed fluctuations in expression of *bdccp* genes during cultivation in vitro, as well as in cultures treated with different drugs and stimuli.

Results: We demonstrated that all *B. divergens* clonal lines tested, originally derived from naturally infected cows, exhibited sexual stages. Furthermore, sexual commitment was stimulated during continuous growth of the cultures, by addition of specific stress-inducing drugs or by alternating cultivation conditions. Expression of *bdccp* genes was greatly reduced or even lost after long-term cultivation, suggesting possible problems in the artificial infections of ticks in feeding assays in vitro.

Conclusions: Our research provides insight into sexual development of *B. divergens* and may facilitate the development of transmission models in vitro, enabling a more detailed understanding of *Babesia*-tick interactions.

Keywords: *Babesia divergens*, Gametocytes, *bdccp* genes, qRT-PCR, Transmission

Background

Babesia are protozoan intracellular parasites infecting various vertebrates including humans. All representatives of the genus are cosmopolitan, tick-transmitted pathogens that belong to the most common blood parasites of mammals [1]. *Babesia* forms a sister clade to *Theileria* and together they form a group referred to as Piroplasmida [1, 2]. Babesiosis caused by *Babesia divergens*, the most common blood parasite in Europe, is a disease in human and veterinary medicine that is occurring with increasing incidence [3]. *Babesia* is evolutionarily related to *Plasmodium* [2], the agent of malaria, and both protists share many features in parasite development, such as asexual multiplication in the red blood cells (RBCs) of the vertebrate host and sexual development in the internal organs of the arthropod vector [4, 5].

Gametocytes represent essential developmental sexual stages of apicomplexan life-cycles and, in the case of *Babesia*, they determine the ability to infect the tick [6, 7]. The commitment from asexual growth to sexual maturation already occurs in the blood stream of the vertebrate host [7, 8]. Unlike *Plasmodium*, *Babesia* gametocytes are barely distinguishable from other asexual stages. For this reason, only laborious electron microscopy has reliably described gametocytogenesis in cultures of *Babesia bigemina* [9] or in the blood of hamsters infected with *Babesia microti* [10]. The only case of gametocyte detection by light microscopy was described after stimulation of *B. bigemina* in vitro by addition of xanthurenic acid

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(XA) [11] or a gut homogenate from fully engorged *Rhipicephalus* (*Boophilus*) *microplus* ticks [12].

Babesia gametocytes are also poorly characterized at the molecular level. Several genes, such as heat shock protein 20 and rhoptry-associated protein 1a were believed to be transcribed in *B. bigemina* sexual stages. However, transcription of these genes was later found not to be exclusive for gametocytes and was also detected in other parasite stages [13]. To date, the only molecular assay enabling specific recognition of *Babesia* sexual stages is based on the analysis of a highly conserved family of proteins named CCp [14]. CCp proteins are, in general, characterized by the presence of at least one Limulus coagulation factor C (LCCL) domain [15, 16] and are often involved in cell adhesion [16]. Gene orthologs from the highly-conserved CCp family have been identified in numerous apicomplexan parasites [16–18], including *Babesia* and *Theileria* species [14, 19]. Transcription of *ccp* genes was found to be restricted to gametocytes in vertebrate blood, while translation occurs in the arthropod vector to mediate gamete fertilization [14, 16, 20–24]. Based on post-genomic bioinformatic analyses of *Babesia* and *Plasmodium* genomes, three *bdccp* genes (*bdccp1*, *bdccp2* and *bdccp3*) were thoroughly characterized and described as markers of *B. divergens* sexual stages [14]. The transcripts of *bdccp1*, *bdccp2* and *bdccp3* genes were also detected in gametocytes appearing in cultures of *B. divergens*, *B. bigemina*, *Babesia bovis* and *Theileria equi* [14, 19]. Moreover, antibody targeted to BdCCp2 protein enabled visualization of *B. divergens* sexual stages exclusively in the midgut of *Ixodes ricinus* [22].

Here, we have established qRT-PCR conditions for the assessment of the efficiency of *B. divergens* gametocytes production in cultures in vitro by measuring the expression of *bdccp* genes. This technique is a unique tool to monitor the kinetics of *B. divergens* sexual stages. We analysed changes in expression of *bdccp* genes following variations in cultivation conditions and identified stimuli that significantly increased gametocytemia. Practical applications of our results have the potential to facilitate further detailed research in the field of *Babesia*-tick interactions.

Methods

Babesia divergens

Strains of *B. divergens* were isolated from bovine blood during the acute phases of babesioses as described earlier [25]. 11 isolates of *B. divergens* from different geographical locations within France were cultivated and cloned by limited dilution [26]. The first two digits in the description of each clone (Additional file 1: Table S1) refer to the French county of origin. Isolate Rouen 87 originated from human blood [27]. *Babesia divergens* isolates were cultivated in vitro in a suspension of bovine erythrocytes obtained from a parasite-free cow (serologically negative and culture tested) as described [25, 26]. Parasitemia was monitored using the commercial Diff-Quik Stain Set (Siemens) and RBC smears.

Selection of target and reference genes, primer design, DNA extraction and PCR

Previously described *B. divergens* gametocyte-specific sequences of *bdccp1*, *bdccp2*, and *bdccp3* (GenBank Accession Nos. FJ943575.1, FJ943576.1, and FJ943577.1, respectively; [14]) were selected as target genes to quantify the presence of parasite sexual stages (gametocytes) in cultures under various conditions (Table 2). Four reference genes were selected: β-tubulin (*b-tubulin*), glyceraldehyde 3-phosphate dehydrogenase (*gapdh*), actin (*actin*) and the small eukaryotic 18S rRNA (*18S*). Sequences were obtained from the *B. divergens* genome database [28] using the nucleotide basic local alignment search tool (BLAST) [29]. All primers were designed using Geneious Pro Trial 5.6.6 software; the sequences and amplicon lengths are summarized in Table 1. The qRT-PCR primers were designed after analysis for polymorphism (see below) particularly towards the conserved regions, especially towards the 3′ end. Negative complementarity of all designed primers with bovine DNA was evaluated *in silico* using BLAST on-line software (blast.ncbi.nlm.nih.gov) and experimentally verified using PCR in a sample containing parasite-free bovine DNA. Genomic DNA (gDNA) was extracted according to the instructions of the Wizard® Genomic DNA Purification Kit (Promega) from frozen infected RBCs as described [30]. PCR was performed using a GoTaq® Flexi DNA Polymerase kit (Promega) with an annealing temperature of 60 °C, using sequencing or qRT-PCR primers according to the manufacturer's instructions.

Polymorphism analysis

Polymorphisms in the selected reference and target genes were evaluated in 11 *B. divergens* clonal lines from different locations in France (listed in Additional file 1: Table S1) and compared to the *B. divergens* genome and other available sequences of *bdccp* genes. Partial gene sequences and amplicons for all target and reference genes were amplified with the sequencing primers, purified with ExoSAP-IT® (USB) and sequenced. Sequences were analysed by BioEdit v7.2.5 software.

Quantitative analysis of expression of *bdccp* genes

Total RNA was extracted by a combination of TRIzol® Reagent (Ambion) and NucleoSpin® RNA extraction kit (Macherey-Nagel). Briefly, 50 μl of pelleted RBCs were mixed with 200 μl of TRIzol and supplemented with 40 μl of chloroform (Sigma-Aldrich), thoroughly vortexed and centrifuged (12,000× *g*, 15 min, 4 °C). The

Table 1 List of oligonucleotides

Gene name and sequence reference	Sequencing primers		qRT-PCR primers	
	Sequence 5′-3′ and amplicon length (bp)		Sequence 5′-3′ and amplicon length (bp)	
gapdh (LK934710)	F: TTGACTGTCGATGGTGCTTC	391	F: TACTTACGAGCAGATCGTTGC	140
	R: ACCATGACACAAGCTTCACG		R: CGGCCTTGACATCGAAAATG	
actin (LK934710)	F: GCTTTGTTACATTGCCCTCG	437	F: GTCAGCGTATGACGAAGGAG	131
	R: CCTCCTTGGTGATCCACATC		R: CTGGAAGGTGGAAAGGGATG	
b-tubulin (LK934711)	F: TTCCCCAGACTGCACTTCTT	400	F: GAGTGGATCCCACACAACAC	138
	R: TGTGTACCAGTGAAGGAAGG		R: CATTGCTGTGAATTGCTCCG	
18S (FJ944825)	–	–	F: ATGCCTAGTATGCGCAAGTC	131
	–	–	R: AAGCCGACGAATCGGAAAG	
bdccp1 (FJ943575)	F: GATCGTTCCTCGCTAGCCTAT	639	F: CGCATGCCAGAAAAACAACC	132
	R: TGCACTGATTTACGCAGCTC		R: GCGTCTTTCAGACATCCTCG	
bdccp2 (FJ943576)	F: GCGGGAGAACATGTAGGATG	701	F: CTGTGAGGCCAACTACTGTG	135
	R: TTCGCAACACAGCTCACAAT		R: AAGTGGTCCACGGTTTTCTG	
bdccp3 (FJ943577)	F: CCCACCTCCTTTGACTTCAG	780	F: GTTGTGGTAAAAGCTGCATGG	139
	R: GTGCATCTTGAGCACGAAAA		R: AGAATCGTGACAACTGCCTC	

aqueous phase (about 100 μl) was mixed with the same volume of 70 % ethanol and loaded onto the NucleoSpin® RNA extraction kit column. Subsequent procedures were carried out according to the manufacturer's instructions. Evaluation of quantity and quality of RNA was performed using NanoDrop (Thermo scientific) and Experion (Bio-Rad) analyses. Residual gDNA was removed by DNase digestion with TURBO DNA-free™ Kit (Ambion) according to the manufacturer's protocol. The absence of residual DNA was verified by lack of amplicon by PCR using qRT-PCR primers for *gapdh*.

Reverse transcription was performed by the SuperScript™III First-Strand Synthesis System for RT-PCR (Invitrogen) using a combination of Oligo(dT) and random hexamers according to the manufacturer's instructions. qRT-PCR assay was performed using HOT FIREPol® EvaGreen® qPCR Mix Plus (Rox) (Solis BioDyne) in the 7300 Real-Time PCR System (Applied Biosystems). For each biological sample, three technical replicates were performed. Each assay included a standard curve generated from triplicate reactions of a 10-fold serial dilution of template. Based on standard curves, reaction efficiency and specificity were verified for each assay and each gene separately; the value of $R2 > 0.98$ (the correlating coefficient obtained for the standard curve) and slopes between -3.58 (reaction efficiency 90 %) and -3.10 (110 %) were accepted [31]. For all genes, the dissociation curve analysis was performed to exclude the formation of primer-dimers and to confirm the specificity of primers. The stability of reference gene expression was tested and evaluated by comparisons of all reference genes.

The qRT-PCR results were analysed using Applied Biosystems 7300 Real-Time PCR instrument software. For analysis of the results, a comparative C_t (cycle threshold) ($2^{-\Delta\Delta Ct}$) method was used [32, 33]. Mean values from technical replicates were assessed and only standard deviation (SD) values ≤ 0.5 were accepted. Target gene expression was normalized using *gapdh* and *actin* and compared using the Student t-test with Welsh's corrections.

Analysis of expression of *bdccp* genes in cultures in vitro

All experiments were designed according to the previously published data for *Babesia* and *Plasmodium* [8, 11, 34–37] and carried out in vitro. All experiments were first assayed as pilot experiments using only single replicates of two *B. divergens* clones (2210A G2 and Rouen G11). Based on the results, experiments indicating fluctuations in *bdccp* transcripts were carried out in biological triplicates using *B. divergens* 2210A G2. Detailed descriptions of all experiments are summarized in Table 2. In addition, expression of *bdccp* genes was analysed in 10 bovine clonal lines from different geographical locations (listed in Additional file 1: Table S1); the quantitative analysis was performed 3 days post (culture) initiation (DPI).

Statistical analysis

Statistical analyses were performed in R (version 3.2.2), a software environment for statistical computing (https://www.r-project.org/), using the Student t-test with Welsh's correction or ANOVA followed by Tukey's multiple comparisons test, assuming that the Bartlett test of homogeneity of variances was passed. Graphs were designed in GraphPad Prism (version 6). For graphical

Table 2 Overview of experimental conditions and resulting effects on expression of *bdccp* genes

Experiment description	*B. divergens* clones	Experiment design	Expression of *bdccp* genes
Continuous culture growth	2210A G2 1802A G8 Rouen G11	The initial parasitemia was set up at 0.1 % and expression of *bdccp* genes was analyzed daily for all five days post culture initiation (DPI); cultivation was performed without medium replacement.	increased*
Long-term cultivation	2210A G2 6903C E2 Rouen F5	*B. divergens* clones 2210A G2 and 6903C E2, were continuously propagated in vitro for ≈ 1 year. Samples before and after long-term cultivation were analyzed; parasitemia was equal for all analysed samples to minimize variations in the expression of *bdccp* genes. Expression of *bdccp* genes by *B. divergens* clone Rouen F5 was analyzed by PCR using gDNA and cDNA.	decreased
Imidocarbe treatment	2210A G2 Rouen G11	The range of efficient doses of both drugs was determined following parasite growth monitoring in vitro for 48 h [66] to select effective concentrations of drugs (imidocarbe 179.5 nM, 359 nM and 718 nM; atovaquone 10 nM, 40 nM and 75 nM). The culture without drug treatment was used as a control. The effect of drug treatment was measured 2 DPI; starting parasitemia was 2 %.	increased*
Atovaquone treatment			increased or decreased* (concentration dependent)
Altered cultivation temperature and air environment	2210A G2	XA was added at 100 μM concentration and its effect was tested after 24 h of parasites cultivation either under standard (37 °C, 5 % CO2) or altered conditions (28 °C, air). As a control, cultures without XA were used. A starting parasitemia was set up 6 % in order to reach > 10 % parasitemia level (experiment design setting taken from [11]).	increased*
XA addition			increased*
Combination of altered cultivation and XA addition			increased*
Co-infection	2210A G2 Rouen G11 7101A D11	Different clonal lines were mixed in the same ratio and expression of *bdccp* genes was analysed in cultures cultivated for 24 h and 48 h. As a control, clones were cultivated independently; starting parasitemia was 2 %.	not affected
RBCs lysate addition	2210A G2 Rouen G11	Lysate of uninfected RBCs was added into the culture to simulate cultivation medium corresponding with 10 % parasitemia. Analyses were performed after 24 and 48 h of cultivation; the control was represented by a culture without lysate addition; starting parasitemia was 2 %.	not affected
Hematocrit increase	2210A G2 Rouen G11	Hematocrit increase was simulated by doubling the quantity of RBCs in the medium and analyses were performed after 24 h and 48 h of cultivation; standard in vitro culture was used as a control; starting parasitemia was 2 %.	not affected
High parasitemia maintenance	2210A G2 Rouen G11	Analyses were performed at the starting point (0 DPI), where parasitemia was starting at 10 %, and 1 and 2 DPI. Media were changed daily.	not affected
Cultivation without FCS	2210A G2 Rouen G11	Altered cultivation conditions (cultivation in medium without FCS) were maintained for 24 h in culture with 10 % parasitemia. Analyses were performed 0 and 1 DPI; starting parasitemia was 2 %.	not affected

Abbreviations: *XA* xanthurenic acid, *RBCs* red blood cells, *FCS* fetal calf serum, *DPI* days post initiation
*$P < 0.05$

representations of the results and statistical analyses, mean values (± standard deviation, SD) from three biological replicates (independent experiments) were assessed.

Results

Analysis of gene polymorphisms

Previously, polymorphisms had not been detected in *18S* rDNA sequences from several *B. divergens* isolates [30]. Hence sequence FJ944825 (GenBank) was used as an *18S* rDNA reference gene. For other reference genes, we did not detect any polymorphisms in the *b-tubulin* gene and only two synonymous substitutions in *gapdh* and *actin* genes. *bdccp1* was found to be highly conserved (one synonymous substitution) compared to *bdccp2* (6 substitutions in the coding regions, 2 non-synonymous, resulting in 5 different sequences) and *bdccp3* (7 substitutions, 2 localized in introns, all synonymous, resulting in 7 different nucleotide sequences) genes (Additional file 2: Figure S1). The qRT-PCR primers were designed only in the conserved regions.

Optimization of expression of *bdccp* genes

qRT-PCR was optimized as recommended by MIQE [31] for reference (*18S*, *gapdh*, *actin*, *b-tubulin* and *18S*), as well as for target (*bdccp1*, *bdccp2*, *bdccp3*) genes. Standard curves of reference, target genes and qRT-PCR parameters are summarized in Additional file 3: Figure S2.

Comparisons between reference genes using C_t values showed that *gapdh* and *actin* were the most stably expressed (Additional file 4: Figure S3) and these genes were therefore selected as references for further analyses. Sample normalization to *gapdh* or *actin* were consistent and no significant differences were recorded.

Expression of *bdccp* genes under standard cultivation conditions

All field bovine clonal lines uniformly expressed *bdccp* genes, with the *bdccp*1 gene having the lowest level of transcripts and the *bdccp3* gene having the highest level (Additional file 5: Figure S4). The influence of long-term cultivation on expression of *bdccp* genes was measured for three clonal lines. The decrease in transcription of *bdccp* genes was also noted in the long-term (≈1 year) cultures of 2210A G2 and 6903C E2 clones (Fig. 1a, b). *B. divergens* clone Rouen F5, propagated in vitro for several years, had already lost the ability to express *bdccp* genes (Fig. 1c). The presence of gametocytes in the original sample of *B. divergens* clone Rouen F5 was confirmed by PCR (data not shown). Asexual multiplication of the parasite was not affected, as demonstrated by the continuous presence of parasitemia in blood smears as well as by expression of the *gapdh* reference gene.

An increase in expression of *bdccp* genes was recorded during continuous growth of all strains of *B. divergens*. Using clone 2210A G2, increased transcription of *bdccp1*, *bdccp2* and *bdcpp3* genes was observed in a pilot experiment (increase 4.7, 4.1 and 3.3 times, respectively; Additional file 6: Figure S5) and confirmed by repeated analysis in biological triplicates, where *bdccp1* and *bdccp2* levels significantly increased from 3 DPI ($F_{(4,\ 10)}$ = 66.02, $P < 0.001$) and 2 DPI ($F_{(4,\ 10)} = 73.85$, $P = 0.033$), respectively. After 5 days of cultivation, *bdccp1* and *bdccp2* gene expression increased 3.5 ($F_{(4,\ 10)} = 66.02$, $P < 0.001$) and 2.7 ($F_{(4,\ 10)} = 73.85$, $P < 0.001$) times, respectively. The level of the *bdccp3* transcript significantly increased only 3 DPI ($F_{(4,\ 10)} = 26.26$, $P = 0.012$) and 5 DPI (2.3 times, $F_{(4,\ 10)} = 26.26$, $P < 0.001$) (Fig. 2a). A similar pattern was observed for *B. divergens* clone 1802A G8, where expression of *bdccp1* and *bdccp2* genes increased significantly 5 DPI: 1.8 ($F_{(4,\ 10)}$ = 11.79, $P = 0.003$) and 2.6 ($F_{(4,\ 10)} = 22.81$, $P < 0.001$) times, respectively. A significant increase in expression of the *bdccp3* gene was recorded from 3 DPI ($F_{(4,\ 10)} = 11.11$, $P = 0.017$) and increased 2.1 times on 5 DPI ($F_{(4,\ 10)} = 11.11$, $P < 0.001$) (Fig. 2c).

Expression of *bdccp* genes under stress conditions

Simulation of stress conditions in *B. divergens* in vitro by drug treatment resulted in a significant increase in expression of *bdccp* genes (Figs. 3 and 4). Imidocarbe, a drug routinely used in veterinary medicine to treat babesiosis [3], almost completely inhibited parasite growth at a concentration of 718 nM ($t_{(4)} = 17.31$, $P <$ 0.001). At this concentration, expression of all *bdccp1*, *bdccp2* and *bdcpp3* genes were significantly increased: 1.8 ($t_{(4)} = -6.36$, $P = 0.004$), 2.5 ($t_{(4)} = -6.96$, $P = 0.007$) and 3.0 ($t_{(4)} = -11.07$, $P < 0.001$) times, respectively, but simultaneously, overall parasitemia was reduced more than 10 times, compared to the control. Treatment with 359 nM imidocarbe showed a moderate killing effect ($t_{(4)}$ = 9.19, $P < 0.001$), but resulted in a significant increase (1.9 times, $t_{(4)} = -9.78$, $P = 0.005$) in expression of only *bdccp3*. 179.5 nM imidocarbe decreased parasitemia by only 1.3 times ($t_{(4)} = 4.40$, $P = 0.018$) with no effect on expression of *bddcp* genes (Fig. 3a, b).

Atovaquone, another effective anti-babesial drug that induces cellular oxidative stress and is commonly used in malaria and human babesiosis treatments [38], caused a significant reduction in growth of *B. divergens* at concentrations of 40 nM (moderate inhibitory effect, $t_{(4)}$ = 17.88, $P = 0.002$) and 75 nM (complete inhibitory effect,

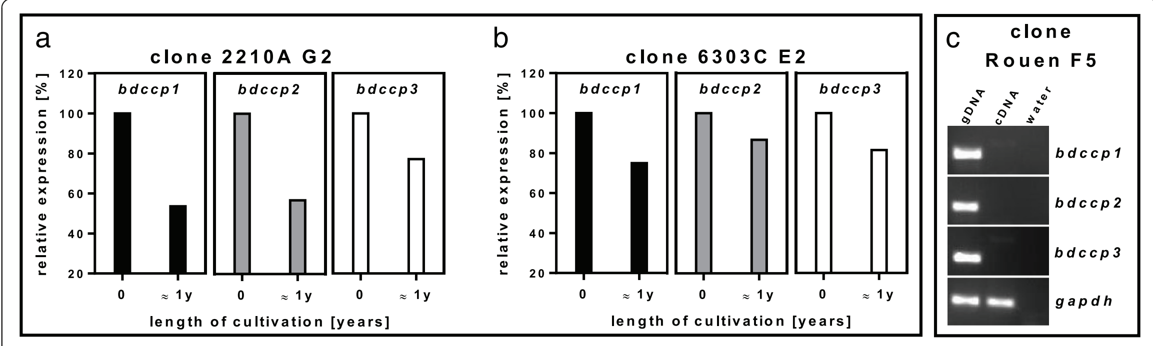

Fig. 1 Long-term cultivation. Influence of long-term [≈1 year (1y)] cultivation on the relative expression of *bdccp* genes during continuous growths of *B. divergens* clones 2210A G2 and 6903C E2 (**a**, **b**). Gene expression was normalized using the *gapdh* reference gene. The expression in samples collected at the beginning of long-term cultivation (0) was set at 100 %. **c** Loss of expression of *bdccp* genes after several years of cultivation of *B. divergens* clone Rouen F5 tested by PCR

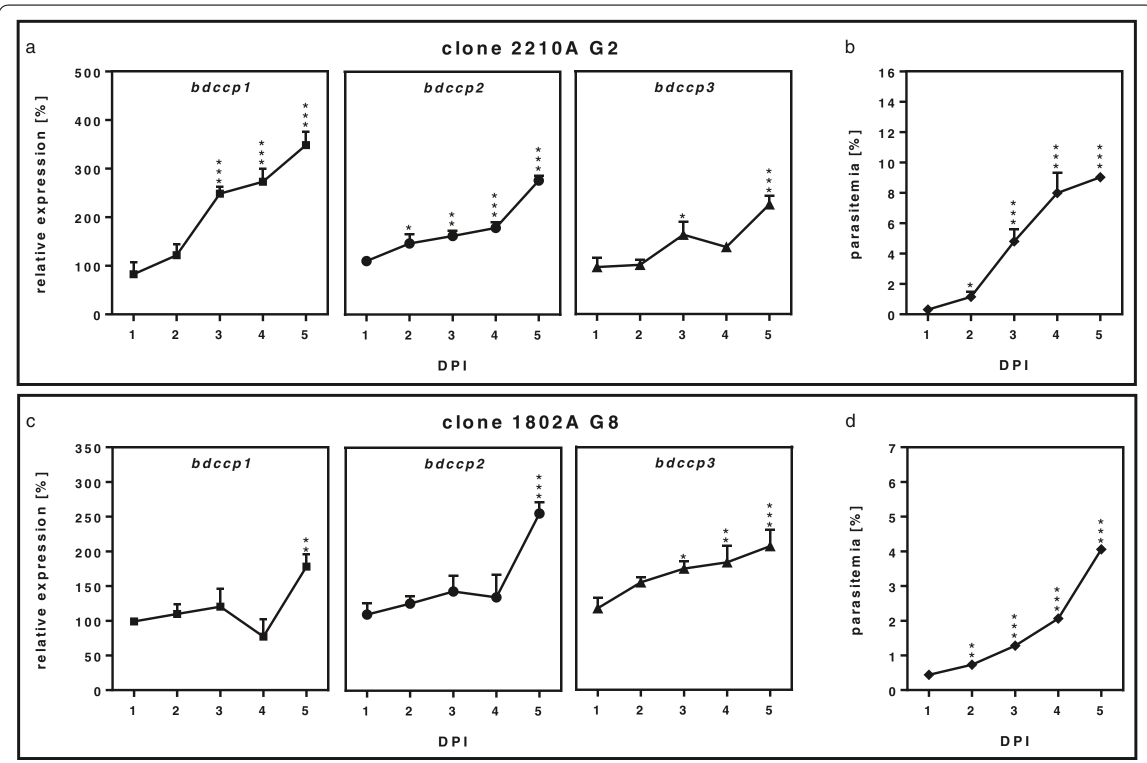

Fig. 2 Continuous culture growth. Relative expression of *bdccp* genes (**a**, **c**) and parasitemia levels (**b**, **d**) during the continuous growth of *B. divergens* clone 2210A G2 and 1802A G8. Gene expression was normalized using the *gapdh* reference gene. The results represent means of three independent biological replicates, where the highest expression in the individual replicate 1 DPI was set at 100 % and all other values were expressed relative to this. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ (compared to 1 DPI). Error bars indicate SD

$t_{(4)} = 22.02$, $P = 0.002$). At a concentration of 40 nM, drug treatment resulted in significantly reduced expression of *bdccp1* and *bdccp2* genes: 2.2 ($t_{(4)} = 4.19$, $P = 0.017$) and 2.0 times ($t_{(4)} = 4.55$, $P = 0.032$), respectively, whereas at 75 nM, atovaquone significantly increased *bdccp3* transcript levels (1.8 times, $t_{(4)} = -4.28$, $P = 0.016$) (Fig. 3c, d).

A reduction in cultivation temperature from 37 to 28 °C in combination with a change in environmental conditions from 5 % CO_2 to an air atmosphere, resulted in significant inhibition ($t_{(4)} = 9.84$, $P = 0.004$) of parasite division as well as in a significant increase in expression of *bdccp1* ($t_{(4)} = -5.57$, $P = 0.005$) and *bdccp2* ($t_{(4)} = -8.32$, $P = 0.002$) genes (Fig. 4a, b). Treatment with XA, a metabolic intermediate of tryptophan degradation, has been proposed to increase the development of sexual stages in *B. bigemina* in vitro [11]. In our experiments with XA treatment and cultivation at 37 °C and 5 % CO_2 we identified conditions that significantly increased expression of all *bdccp1*, *bdccp2* and *bdcpp3* genes: 1.9 ($t_{(4)} = -3.97$, $P = 0.017$), 2.4 ($t_{(4)} = -11.97$, $P < 0.001$) and 1.6 ($t_{(4)} = -5.27$, $P = 0.006$) times, respectively (Fig. 4a) without any inhibitory effect on culture growth (Fig. 4b). Combining XA treatment with altered cultivation conditions (28 °C, air atmosphere) resulted in significantly increased expression of *bdccp1* (2.0 times, $t_{(4)} = -3.54$, $P = 0.029$) and *bdccp2* (2.4 times, $t_{(4)} = -8.39$, $P = 0.001$) genes but culture growth was significantly inhibited ($t_{(4)} = 10.80$, $P = 0.002$) (Fig. 4b). All other stress factors tested did not result in a significant increase in expression of *bdccp* genes (Table 2).

Discussion

The production of gametocytes in the host blood is a prerequisite for successful parasite transmission to the arthropod vector. *Plasmodium* gametocytemia, which could be quantified by simple light microscopy [39, 40], was demonstrated to closely correlate with mosquito infection [41–44]. However, such a simple morphological identification is not possible for *Babesia* gametocytes, preventing controllable infections of ticks. Based on similarities between these two parasites [2], we presumed that similarly to *Plasmodium*, changes in the expression of *Babesia* sexual stage-specific *bdccp* genes would correlate with actual numbers of gametocytes in

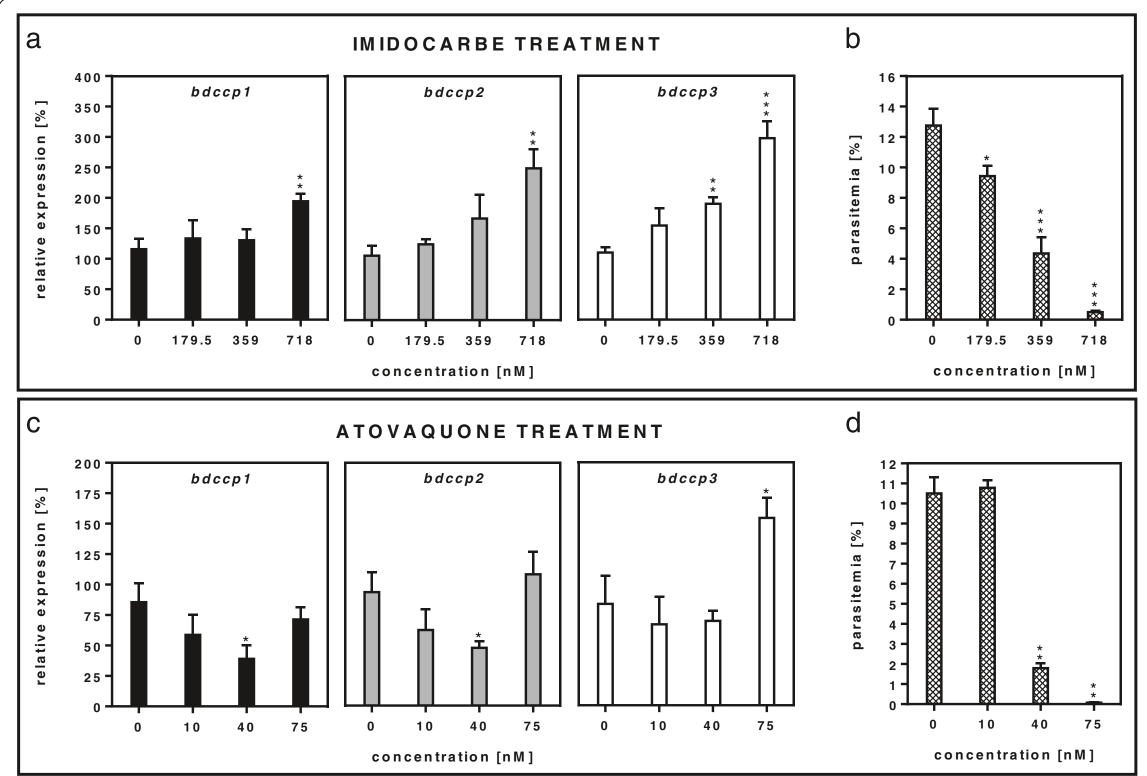

Fig. 3 Imidocarbe and atovaquone treatment. The effect of imidocarbe (**a**) and atovaquone (**c**) treatment on the relative expression of *bdccp* genes and corresponding parasitemia (**b**, **d**) of *B. divergens* clone 2210A G2. Gene expression was normalized using the *gapdh* reference gene. The results represent means of three independent biological replicates, where the highest expression in the individual replicate of untreated culture (0) was set at 100 % and all other values were expressed relative to this. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ (compared to the untreated culture). Error bars indicate SD

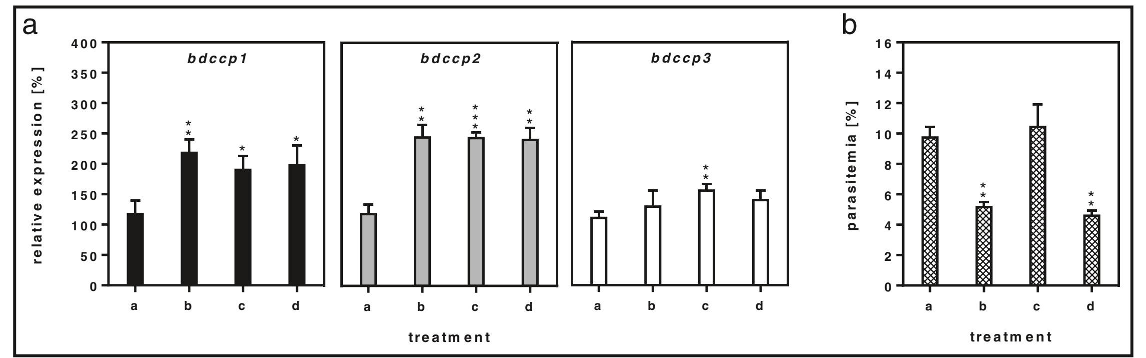

Fig. 4 XA treatment and altered cultivation conditions. The effect of XA (xanthurenic acid) treatment and altered cultivation conditions on the relative expression of *bdccp* genes (**a**) and corresponding parasitemia levels (**b**) of *B. divergens* clone 2210A G2 culture. (*a*): untreated culture cultivated at 37 °C, 5 % CO_2; (*b*): untreated culture cultivated at 28 °C, air; (*c*): XA treated culture cultivated at 37 °C, 5 % CO_2; (*d*): XA treated culture cultivated at 28 °C, air. Gene expression was normalized using the *gapdh* reference gene. The results represent means of three independent biological replicates, where the highest expression in the individual replicate of untreated culture (*a*) was set at 100 % and all other values were expressed relative to this. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ (compared to the untreated culture). Error bars indicate SD

the total intra-erythrocytic parasite population. Using previously described sequences of *bdccp1*, *bdccp2* and *bdccp3* genes [14], we have developed a qRT-PCR assay to detect and quantify gametocyte densities in *B. divergens* cultures in vitro. Based on comparisons between the reference genes we chose *actin* and *gapdh* as references for our assays (Additional file 4: Figure S3). The *18S* rDNA exhibited lower stability than *actin*, *gapdh* or *b-tubulin*. This result differs considerably from the generally accepted view that *18S* rDNA is one of the most stably expressed genes [45, 46].

The selection of specific target and reference gene primers, universal for most of the *B. divergens* strains, was absolutely critical for further reliable assessment of the gametocytes production efficiency by qRT-PCR. Despite the fact that CCp proteins are presumed to be conserved among the apicomplexan parasites [16, 19, 20], no data were available about single nucleotide polymorphisms of *ccp* genes among various strains within one species. We demonstrated that between the 11 *B. divergens* clonal lines, nucleotide sequences of *ccp* genes varied, especially for *bdccp2* and *bdccp3* genes (Additional file 2: Figure S1). On the contrary, the *bdccp1* gene was highly conserved. The sequences of reference genes seemed to be highly conserved. Some studies questioned the suitability of *actin* and *gapdh* reference genes because of their variabilities [45], but our results did not support this (Additional file 2: Figure S1) and confirmed their suitability.

The appearance of gametocytes in the blood is a crucial event that it is still not fully understood. Referring to the recent knowledge on *Plasmodium*, commitment towards the sexual development occurs randomly, asynchronously and is governed by the genetic and environmental factors [47], as demonstrated by detailed studies performed on *Plasmodium* (see reviews [8, 36, 37, 48–50]). To date, only one study has been dedicated to this subject in *Babesia* (*B. bigemina*) [11].

We tested the effects of various factors and conditions on gametocytogenesis in *B. divergens* cultures. Our results demonstrated the ability of *B. divergens* to produce gametocytes (measured by expression of *bdccp* genes in several bovine strains; Additional file 5: Figure S4) after a short term cultivation. On the contrary, long-term cultivation led to a significant decrease or even absence of expression of *bdccp* genes (Fig. 1), suggesting that these cultures had halted production of gametocytes and were probably no longer infectious for ticks. Similarly to *Babesia*, the disappearance of gametocytes from long-term maintained *Plasmodium falciparum* cultures has also been described (reviewed in [37]), therefore only fresh cultures with low passage numbers should be used for tick or mosquito infection studies.

The enhancement of *Babesia* sexual commitment was observed after several days of cultivation without medium changes, but minor variations were recorded in the *bdccp* genes expression of various *B. divergens* strains (Fig. 2, Additional file 6: Figure S5). Such phenomena could be explained by the stochastic differentiation mechanism, that was previously reported for *Theileria* [51]. A rapid expansion of a *Plasmodium* population (intensive multiplication of asexual stages) also resulted in an increase in gametocytogenesis [34, 35]. A possible explanation of this phenomenon is the accumulation of metabolites under stress conditions, as high parasitemia or regular medium exchanges did not alter levels of *bdccp* transcripts (Table 2). This change is probably induced by the accumulation of metabolic waste in the blood, as an addition of a lysis solution of healthy RBCs had no significant effect (Table 2). Nevertheless, hemolysis products of both infected and healthy RBCs influenced production of gametocytes of *P. falciparum* in vitro as well as *Plasmodium chabaudi* in vivo [52, 53]. As previously shown, mixed population of *Plasmodium* species could result in an increase of gametocytemia and promoted more successful transmission into the vector [36, 54, 55] despite some contradictory results [56]. We did not observe this phenomenon for *B. divergens* isolates (Table 2), however the choice of strains could greatly influence results, depending on their modes of interaction (neutral or synergistic instead of antagonistic).

Addition of inhibitory drugs certainly represents a stressful condition for the parasite. Numerous experiments performed on *Plasmodium* proved that treatment with anti-malarial drugs had an effect on the recruitment of gametocytes, both in vivo and in vitro (reviewed in [8, 48, 50, 57]). We have tested the effects of imidocarbe and atovaquone, the widely used anti-babesial drugs. Imidocarbe has been used for over 20 years as the drug of choice for the treatment and prophylaxis of animal babesiosis [58]. The mode of action of imidocarbe still remains unclear, although disruption of polyamine metabolism or a blockage of inositol influx into parasitized cells was proposed [58]. In our experiment, imidocarbe treatment significantly increased all three *bdccp* transcripts, while overall parasitemia was greatly decreased (Fig. 3). This implies that this drug either stimulated sexual commitment to the sexual pathway or has a lower impact on gametocytes as they are metabolically less active compared to asexual stages.

Atovaquone is widely used to treat babesiosis (and malaria) in humans [38] and causes oxidative stress in the parasite by inhibition of the mitochondrial electron transfer [59]. This drug displayed remarkable activity against asexual stages. In gametocytes, only *bdccp3* gene transcription was significantly increased (Fig. 3). It was previously demonstrated that atovaquone treatment had different effects on the various maturation stages of *P. falciparum* gametocytes [60, 61]. Therefore, we speculate that differences in gene expression of *bdccp1* and *bdccp2*

compared to *bdccp3* upon atovaquone application could be also related to the age of *Babesia* gametocytes.

Physical or chemical alterations of the parasite environment that mimic transition from the blood stream to the vector gut (temperature decrease from 37 to 28 °C, CO_2 decrease from 5 % to air environment and addition of a gut homogenate from fully engorged ticks or XA) have been shown to have an effect on the *Babesia* sexual development [11, 12, 62]. We observed a similar stimulation of *B. divergens* sexual commitment after changes in the cultivation environment and/or XA addition using analysis of *bdccp* genes transcription. However, no apparent cumulative effect was observed when combining these stimuli. We demonstrated that XA addition into the culture under standard cultivation conditions (37 °C and 5 % CO_2) significantly stimulated *B. divergens* sexual commitment without inhibiting parasite growth. This is in contrast to previously published results for *B. bigemina*, where no change in gametocyte development occurred upon XA treatment of a culture propagated under the same conditions [11]. In mosquitoes, XA naturally produced inside the gut is able to induce gamete formation and exflagellation of *Plasmodium* parasites [63, 64]. As exflagellation does not occur in the *Babesia* life cycle, the exact effect of XA on *Babesia* sexual development remains to be elucidated. It is not verified yet whether XA is produced inside the tick gut. If so, one can speculate that *Babesia* gametocytes might be stimulated in the host blood by the tick gut contents regurgitated during the week-long feeding of the adult tick female. This hypothesis could be supported by the studies demonstrating that gametocyte development was stimulated after addition of tick gut homogenate [12, 62]. Further investigation is needed to provide an unequivocal answer.

Conclusion

Compared to *Plasmodium*, sexual development of *Babesia* is poorly understood. Our research provided insight into sexual development of *B. divergens* during either standard cultivation conditions in vitro or cultivation under stress by different stimuli. Using our newly introduced quantification assay of *bdccp* genes transcripts by qRT-PCR we have shown that levels of gametocytes fluctuate during *B. divergens* culture in vitro and identified conditions that significantly increased the transcription of *bdccp* genes (and thus gametocytemia). By setting these conditions we should be able to perform studies focusing on the transmission and persistence of *Babesia* in the tick vector using an artificial membrane feeding system of ticks [65]. Research aimed to identify and characterize molecular mechanisms of interaction between the parasite and the tick vector could accelerate discovery of effective therapies or vaccines blocking *Babesia* transmission.

Additional files

Additional file 1: Table S1. List of *B. divergen*s strains used in this study (a – gene polymorphism analysis, b – analysis of expression of *bdccp* genes). (DOC 37 kb)

Additional file 2: Figure S1. Consensus partial nucleotide sequences of genes used for design of qRT-PCR primers. (A) *gapdh*, (B) *actin* (C), *b-tubulin*, (D) *bdccp1*, (E) *bdccp2*, (F) *bdccpp3*. The localization of introns and primers are indicated in yellow and blue, respectively. The sequences were obtained from 11 different biological clones from France: Rouen F5 (human origin), 1406B F10, 1505B F4, 1705A G10, 2705A E11, 3601B E2, 4201B D4, 4903A D11, 5012A G3, 7904B G11, 8706A G8 and from the *B. divergens* genome sequence. As previously reported, sequencing of the *18S* rDNA revealed no variation within *B. divergens* [30], so sequence FJ944825 was taken as reference. Variable nucleotides are highlighted and the corresponding clone(s) and modifications are indicated below each consensus sequence using a color code. (PDF 10 kb)

Additional file 3: Figure S2. Optimization of qRT-PCR. Standard curves of reference and target genes (A) and qRT-PCR parameters (B). C_t = cycle threshold, R2 = correlation coefficient. (PDF 181 kb)

Additional file 4: Figure S3. Comparison of stability of reference genes. Reference genes were evaluated by comparisons of all reference genes using C_t values. The first sample in each gene analysis was set at 100 % and all other values were normalized to this. (PDF 66 kb)

Additional file 5: Figure S4. Relative expression of *bdccp* genes in various bovine clonal lines of *B. divergens*. Gene expression was normalized using the *gapdh* reference gene. Expression in the clone 2210A G2 was set at 100 % and all other values were expressed relative to this. (PDF 49 kb)

Additional file 6: Figure S5. Continuous culture growth. Relative expression of *bdccp* genes (A) and parasitemia levels (B) during the continuous growth of *B. divergens* clone 2210A G2. Gene expression was normalized using the *gapdh* reference gene. The expression in the highest individual replicate 1 DPI was set at 100 % and all other values were expressed relative to this. (PDF 48 kb)

Abbreviations

BLAST, basic local alignment search tool; bp, base pairs; cDNA, complementary DNA; C_t, cycle threshold; DPI, days post (culture) initiation; FCS, fetal calf serum; *gapdh*, glyceraldehyde 3-phosphate dehydrogenase; gDNA, genomic DNA; LCCL, Limulus coagulation factor C; qRT-PCR, quantitative real-time PCR; RBCs, red blood cells; SD, standard deviation; XA, xanthurenic acid

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Availability of data and materials

The datasets supporting the conclusions of this article are included within the article and its Additional files.

Authors' contributions

Conceived and designed the experiments: MJ, CB, LM. Performed the experiments: MJ. Analysed the data: MJ, CB, OH, PK, LM. Contributed

reagents/materials/analysis tools: CB, OH, PK, LM. Wrote the paper: MJ, OH, PK, LM. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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Not applicable.

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Additional file 1: Table S1.

List of *B. divergens* strains used in this study (a – gene polymorphism analysis, b – analysis of expression of *bdccp* genes).

Strain	County in France	Use
Rouen 87 (clones F5 and G11)	Seine Maritime	a-b
1406B F10	Calvados	a-b
1505B F4	Cantal	a
1705A G10	Charente-Maritime	a
1802A G8	Cher	b
2210A G2	Côtes d'Armor	b
2305B E7	Creuse	b
2705A E11	Eure	a-b
3601B E2	Indre	a
4201B D4	Loire	a
4903A D11	Maine et Loire	a
5005A G5	Manche	b
5008A D10	Manche	b
5012A G3	Manche	a
5608A D10	Morbihan	b
6903C E2	Rhône	b
7101A D11	Saône et Loire	b
7904B G11	Deux Sèvres	a
8706A E8	Haute Vienne	a

A- GADPH partial gene consensus sequence (nt 666157 to 665789 in LK934710)

GGTGCTTCCCGCGGTGGCAAGGACTGGAGGGCTGGTCGGTGCGCCGGTGCCAACATCATCCCTGCTTCCACTGGTGCCGCCAA
GGCTGTTGGCAAGGTCATTCCTGAGTTGAACGGCAAGCTCACTGGTATGGCTTTCAGGGTACCTACTCCCGATGTCAGTGTTGT
TGACTTGACCTGCAAGCTTGCTAAGCCAGCTACTTACGAGCAGATCGTTGCTGCTGTCAAGGAGGCTGCTGAGGGTGAGCTTA
AGGGTGTGTTGGGCTGGACCGAGGACCAGCTGGTATCCCAGGACTTCGTGCACGACCGCAGGTCTAGCATTTTCGATGTCAAG
GCCGGTATTGCTCTGAACGACACCTTCGTGAAGCTT

A in 1705A G10; G in 4201B D4

B- Actin partial gene consensus sequence (nt 1289265 to 1288852 in LK934710)

TGCCCTCGACTTCGAGGAGGAGATGAACTCTGCCTCCTCATCCAGTGAGATTGAGAAGTCGTACGAGTTGCCTGATGGAAACAT
CATCACTGTTGGCAACGAGCGGTTCAGGTGCCCTGAGGTGTTGTTCCAGCCCACCTTCATTGGTATGGAGTCTCCTGGTATCCA
CACCACAACCTTCAACTCCATTGCCCGCTGTGATCTCGACATCCGCAAGGACCTCTACTCAAACGTCGTGTTGTCTGGAGGTAC
GACGATGTACGAGGGTATCGGTCAGCGTATGACGAAGGAGCTGAATGCCTTGGTTCCCAGCACCATGAAGATCAAGGTTGTTG
CCCCTCCGGAGCGGAAGTACTCTGTCTGGATTGGTGGTTCGATTCTATCATCCCTTTCCACCTTCCAGCAGATGTGGAT

C in 1705A G10 ; C in Rouen F5, 4903A D11, 8706A E8 and 1705A G10

C- Beta Tubulin partial gene consensus sequence (nt 833653 to 833244 in LK934711)

TGCACTTCTTCATGATTGGATTCGCACCACTCACGAGCAGGGGAAGCCAGCAGTACCGTGCCCTCACGGTCGCCGAACTTACA
CAGCAAATGTTCGACGCCAAGAACATGATGTGTGCGTCTGACCCCAGGAGGGGGAGGTATCTCACAGCCTGCGCCATGTTCAG
AGGGCGCATGAGCACCAAGGAAGTCGACGAACAGATGTCGATGGTCCAGAACAAAAACGCCTCATACTTTGTGGAGTGGATCC
CACACAACACAAAGTCAAGCGTATGTGACATCCCACCAAAGGGGCTCAAGATGGCCGTTACATTCGTAGGAAACTCAACCGCC
ATCCAGGACATGTTCAAAAGGGTATCGGAGCAATTCACAGCAATGTTCAGGCGTAAGGCCTTCCTTCACTGGTACACA

D- BdCCp1 partial gene consensus sequence (nt 4504 to 5124 in FJ943575.1)

CTCGCTAGCCTATTTAAGGCTGCTGATTATGAATGCAAGGCAGATATCACTGACGACAAAACGCTGGGATTCAACCTGGGGCTC
AAGTCCAGTGCGTGCGACTCTTGCTACTTTGACACATTCCAGGTATCACCAGACCCAATTCAGACCGTTGTACCAGCGACTAAT
TTCGTCGAAGAGTTCTCCAGTGCCAATGTATGGAAGCCATGCGTGGAAAACGTCCATATATTAAGCAGAGTGGCATTATGCAAG
CAGATGTATCGTAATAAAGGAAACTCGCACGTCTGTGCAGAAAGTTTCTGTACCCCTTGTTGCGCCTATCACACTGTGCTGCTG
GGAGACGGGCATCAGCAAATGTGTGTCGACGCATGCCAGAAAAACAACCACCGTGTGAACGCTTACCTAGAGAATTTCCTGTC
GTACGTCAACAGCTGTGTGTCGCTAAAGGGGGTGGGATACGATCACTGTGATGGGGTAAGTATGGCGCCAAATAGCTTGTTCAT
AATGCTGTAGGATCGAGGATGTCTGAAAGACGCATGCAGACTTTGCTGCACCAGTGGGCACCAGGAGAACAGCCTGCTTGACA
AGCAACTTGTGCTTTTGGAAACGTCGAGCTGCGTA

T in 8706A E8

E- BdCCp2 partial gene consensus sequence (nt 4336 to 5018 in FJ943576.1)

CATGTAGGATGATTCAGATGCACAAGGGTAGCCGCATCGTCCTAGCGGAAACTTCCATGAAGGGAATGGTCATCGGTGCGTGGA
ATACCGTTGAACTGGTCTTCAAACCTGACGCTGTAACTGCTTCTATCGGCACTAATCTACTAGAGGCAGTGTTCATTAACACAGC
CGTGGCAGAGGATGTGCGTGAGCGCGGAACTGTCGGCATTTACAGTCTTGGCTGTGCGGGGTGTGCCTTCGCTGGTATCTCACT
TACCCCCAACTACGCCGTCGGGTATGCCAAGATGCTGACCAGCTCTGAGAACGCCAACACGGGTAGCAGGTCGGCACGGGAG
GAGCAGTGCATGGCAATCGACCGCCTGGACCATTGCAAGGCCATCGCATCAGCCAACGTGGCAAACTGTGAGGCCAACTACTG
TGCCTTCTGCTGCGAACAGCAGCATAGGGATGCACCAGGGCCTCAGGATGTGTGTTACAACAGATGCAGAAACTTAGATCATGC
GGCCGTGCTACTGCAGAAAACCGTGGACCACTTCTGGCGCAGCTGCGCACCGAATAGCAACAGCGAAACAAATGATCAAAAA
GGTGGGTTCAATGACTTAGATATAATGCGTAACATTACTCTCACAGGATCAGTTGGTGCAATCGAATACGGAACCGATGCTGTGG
GGGATTGTGAGCTG

T in FJ943576.1; G and T in 1406B F10, 1505B F4, 1705A G10, 7904B G11 and FJ943576.1; C and C in 1406B F10, 1505B F4, 1705A G10, 4903A D11, 7904B G11 and FJ943576.1; G in 1705A G10

Non synonymous substitutions : A (Thr) to G (Ala), T (Ser) to C (Pro).

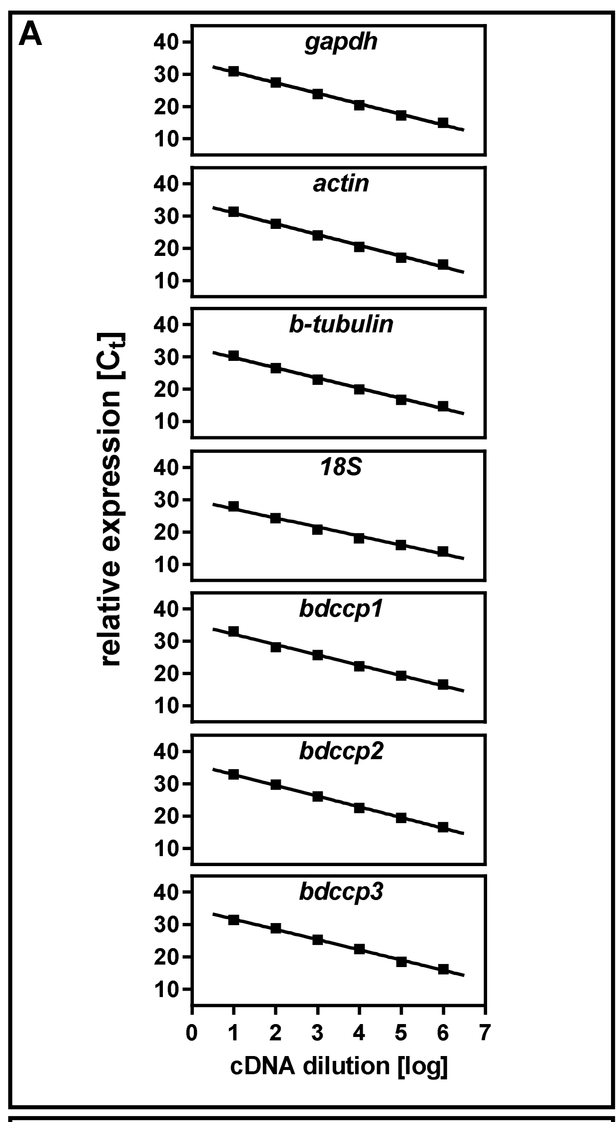
F- BdCCp3 partial gene consensus sequence (nt 3022 to 3780 in FJ943577.1)

TGACTTCAGTTTGCAGGTTTGGTCTGTCTTTTGCGTGGTCTATACTTGTTTAGGGCGATTTAGTCCTTGGGAAGTCTGCGGAGAT
TGATTCTGACTTTTTTATCGGCCAGATCCTGTCGCTGGAGATTTTCGATTACGTCATGTCTCCAGATCAGGTGGAGCACTACAGC
CGTCTGTTACAGGCTTCTAGCGACAATCGGATCAACTCTCGCCACTCTCCAACTCGTATGACTGTTGATGGCAATCTGTGTCTGA
GCAGCTGCGTGAAGATGCGCGCTCCATCGCAAAACGCGGGTTTGCTTGCGGGCCCTACCAATCCTGCTATCCATTTGGGTTGCC
ACGATTCGCTTGAGAACGAGCGTTTCAATGGCGCTACTGGGCATGAGTTTTTGGTGAGTTGTGGTAAAAGCTGCATGGACCCTG
ATTTGGTCCTTAACGGCAGCAAGGTGTACACCCCCGACTCTTCTATATGCAAGGCTGCCATCCACTGTGGTGCGATTCCCAAGG
AAGGCGGCGAGGCAGTTGTCACGATTCTACATGGTCGCGATAGCTACGACCACTGCCTGGGTCATTACGGTAGGTGCACTTTAG
ATTCTACATTCATACGCTTCAGGGGTGCTGAGCAACAAGTCTAGCATTCCCCATATGCGTTCGTTTTCTGTGCGCAGAGCCCCCA
AGGTGTTGACTCTGTCGTGTCAAGGTAAGCGTGCCGATTTGTGTTTCTGTATGACTGCTTGCAGACACCGCTATTTTCGTGCT

T in 1705A G10, 4201B D4, 5012A G3, and 7904B G11; T and T in 3601B E2, 5012A G3 and 8706A E8 ; G in FJ943577.1 ; A in 3601B E2 and 8706A E8 ; C in 4903A D11 ; T in 4201B D4.

Additional file 2: Figure S1.

Consensus partial nucleotide sequences of genes used for design of qRT-PCR primers. (A) *gapdh*, (B) *actin*(C), *b-tubulin*, (D) *bdccp1*, (E) *bdccp2*, (F) *bdccpp3*. The localization of introns and primers are indicated in yellow and blue, respectively. The sequences were obtained from 11 different biological clones from France: Rouen F5 (human origin), 1406B F10, 1505B F4, 1705A G10, 2705A E11, 3601B E2, 4201B D4, 4903A D11, 5012A G3, 7904B G11, 8706A G8 and from the *B. divergens* genome sequence. As previously reported, sequencing of the *18S* rDNA revealed no variation within *B. divergens* [30], so sequence FJ944825 was taken as reference. Variable nucleotides are highlighted and the corresponding clone(s) and modifications are indicated below each consensus sequence using a colour code.

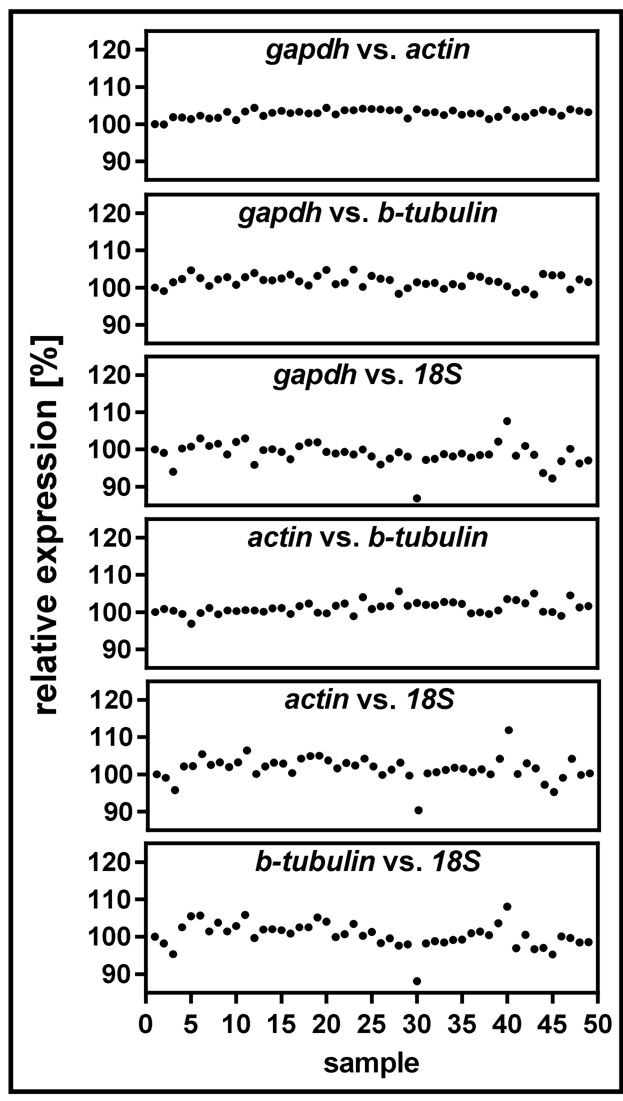


B

gene	R2	slope	efficiency [%]
gapdh	0.994	-3.182	106.171
actin	0.994	-3.261	102.616
b-tubulin	0.991	-3.161	107.203
18S	0.994	-3.182	106.214
bdccp1	0.990	-3.199	105.413
bdccp2	0.998	-3.318	100.147
bdccp3	0.997	-3.160	107.227

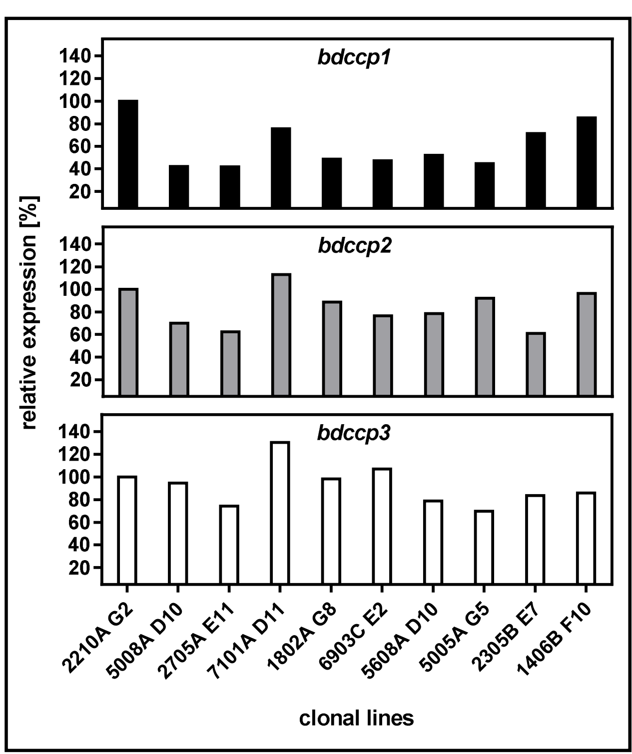
Additional file 3: Figure S2.

Optimization of qRT-PCR. Standard curves of reference and target genes (A) and qRT-PCR parameters (B). C_t = cycle threshold, R2 = correlation coefficient.



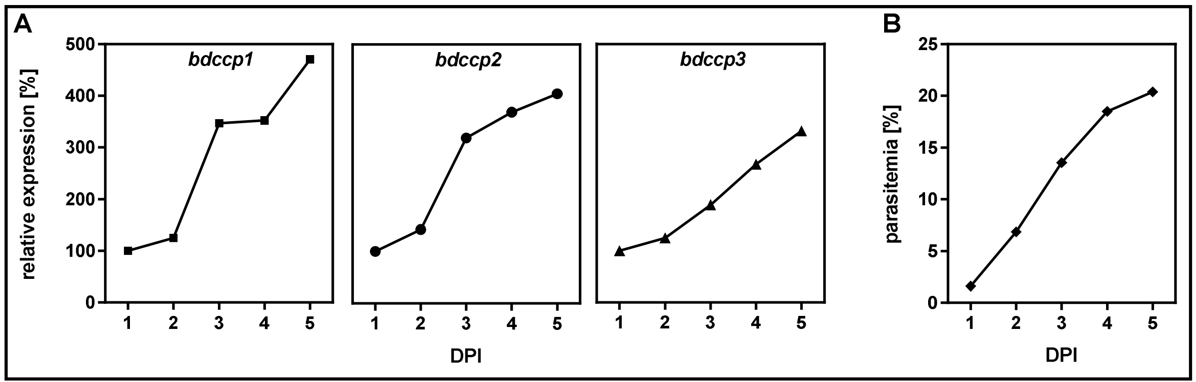
Additional file 4: Figure S3.

Comparison of stability of reference genes. Reference genes were evaluated by comparisons of all reference genes using C_t values. The first sample in each gene analysis was set at 100 % and all other values were normalized to this.



Additional file 5: Figure S4.

Relative expression of *bdccp* genes in various bovine clonal lines of *B. divergens*. Gene expression was normalized using the *gapdh* reference gene. Expression in the clone 2210A G2 was set at 100 % and all other values were expressed relative to this.



Additional file 6: Figure S5.

Continuous culture growth. Relative expression of *bdccp* genes (A) and parasitemia levels (B) during the continuous growth of *B. divergens* clone 2210A G2. Gene expression was normalized using the *gapdh* reference gene. The expression in the highest individual replicate 1 DPI was set at 100 % and all other values were expressed relative to this.

Appendix to publication "Stimulation and quantification of *Babesia divergens* gametocytogenesis"

Initially, we intended to visualize *B. divergens* gametocytes because the commitment to sexual maturation already occurs in the blood stream of the vertebrate host [16, 175]. Unlike *Plasmodium*, *Babesia* gametocytes are hardly distinguishable from other asexual stages, merozoites and trophozoites. To date, only laborious electron microscopy represents the most reliable technique to study *Babesia* sexual stages, as was documented for *B. bigemina* and *B. microti* [46, 176]. Using light microscopy, supposed sexual stages were insinuated only for *B. bigemina* after stimulation [45, 177]. In order to visualize *B. divergens* gametocytes in bovine red blood cells, we introduced and optimized the technique of fluorescence *in situ* hybridization (FISH) targeting to mRNA of *bdccp2* gene. The visualization based on mRNA staining resulted from the natural behaviour of *bdccp* genes, which are translated exclusively in the environment of tick midgut lumen [142, 143]. Yet, although we succeeded with the establishment of a new method in *Babesia* spp. research (Fig. 10), we were not able to reliably distinguish gametocytes in parasitized *in vitro* cultivated bovine red blood cells. Based on discussion with dr. Juan Mosqueda, studying *B. bigemina* gamecytogenesis [177], we hypothesizes that the mRNA abundance of gametocytes is very low *in vitro* cultures, which correlates with previous observations [45, 177]. Due to negative visualization of *B. divergens* gametocytes the technique was not included in the publication but we use it in the further research with very promising results (see Part IV., Fig. 15).

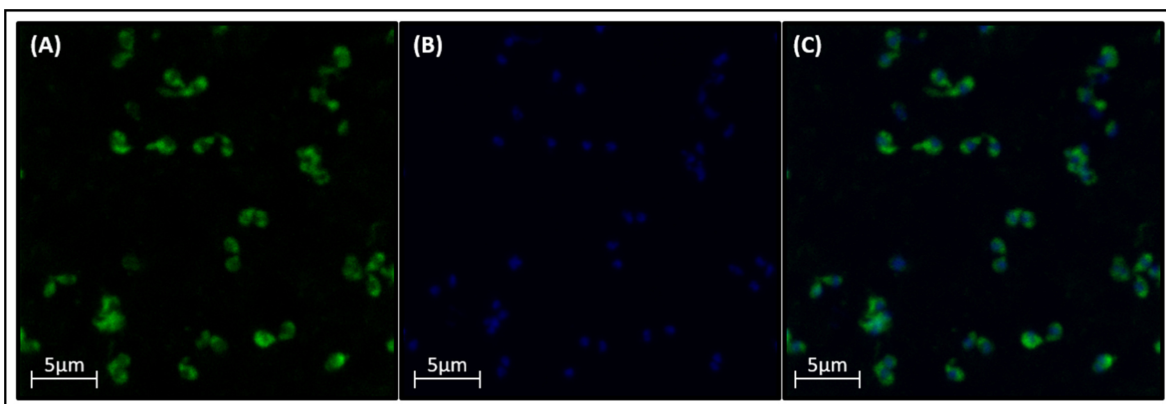


Figure 10. *B. divergens* in bovine erythrocytes stained by fluorescence *in situ* hybridization (FISH). The *B. divergens* parasitized bovine red blood cells underwent the staining procedure by Alexa 488 labeled probes specific to parasites rRNA (A, C) (*18S*; green color; the staining procedure is described in Part II.; the probes sequences are specified in Part II., Tab. 2). Nuclei of cells (blue color) were stained by DAPI (B, C) (4',6-diamidin-2-fenylindol). After the staining procedure, the samples were examined by confocal microscope Olympus FW1000 under the magnification 100× - 6000×. Photos were processed in Fluoview software (FV10-ASW, version 1.7). (A) *B. divergens* blood stages stained by 18S rRNA targeting probes. (B) Cells nuclei staining by DAPI. (C) Merged picture.

Manuscript in preparation "Validation of *Babesia* proteasome as a drug target"

As mentioned previously, babesiosis is an emerging disease with great veterinary and medical impact [4-6]. Despite the growing incidence of *Babesia*-caused diseases, the specific anti-babesial therapeutic agents are used only in veterinary medicine [178]. Human babesiosis is usually treated by anti-malarial and anti-bacterial drugs [6] but these drugs are usually associated with major side effects and even drug failure due to increase of resistant parasites [73, 178, 179]. Thus, the development of new anti-babesial drugs with low toxicity is highly desirable.

Currently, great attention is paid to recently documented anti-parasitic efficacy of proteasome inhibitors [180-187]. Proteasome is large multi-component protein complex involved in regulation of many cellular processes [188] and exhibit a vital importance for the development and survival of parasitic organisms [186]. Drugs from the family of proteasome inhibitors were recently reported as powerful strategy for malaria, leishmaniosis, sleeping sickness and Chagas disease treatment with a remarkable low toxicity to mammalian hosts [180-185].

Therefore, we took an advantage of previously established *B. divergens in vitro* cultures in our laboratory and conducted the primary screening on effect of selected commercially available compounds targeting babesial proteasome. Our results, thoroughly described in the manuscript in preparation (pages 93-108), validated the proteasome inhibitors as potential viable anti-babesial drugs and offer a promise as next-generation of anti-babesial agents.

1 **Validation of *Babesia* proteasome as a drug target**

2

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16 **Keywords:**
17 Proteasome, *Babesia*, Carfilzomib

18

19 **ABSTRACT**

20 Babesiosis is an emerging zoonosis caused by the apicomplexan parasites of the genus *Babesia*
21 spp., closely related to malarial *Plasmodium*. Empirical and consensual treatment of this disease
22 has been mostly based on antimalarial drugs and antibiotics. We tested here the potency of selected
23 commercially available compounds targeting the 20S proteasome catalytic subunits to the growth
24 and survival of *Babesia divergens* in *ex vivo* cultures. We obtained significant suppressive effects
25 growth including the IC_{50} determination for each tested compound. We revealed a remarkable
26 selectivity of the tested compounds for parasite cells. Effective concentrations needed to
27 specifically inhibit the parasite were significantly lower than the toxic concentrations affecting host
28 cells. Furthermore, we used the most promising candidate – carfilzomib - to significantly reduce
29 parasite multiplication in *Babesia microti* infected laboratory mice *in vivo* without any apparent
30 effects to the host. Overall, our results validate proteasome inhibitors as potentially viable anti-
31 piroplasmidal drugs that are worth of further experimental development resulting in even better
32 selectivity of novel compounds for highly-effective anti-babesial treatment.

33

Pages 94-108 were removed because they contain unpublished data. If you are interested, please contact jalovecka@paru.cas.cz.

PART IV. Establishment of *Babesia microti* laboratory model and its experimental application

Part IV. is summarizing all experiments performed in order to introduce complete laboratory model(s) of *B. microti* (Chapter 1. Establishment of *B. microti* laboratory model). Subsequently, the established model was used to investigate the immune interactions between *B. microti* and *I. ricinus* ticks (Chapter 2. Effect of tick immune genes on *B. microti* acquisition). The obtained results are thoroughly described and discussed to point out their interest for the scientific community.

In this part, no publication is included but the described data serve as essential background for intended publication(s) once the experiments are completed. At the end of this part, the additional experiments are indicated.

The part of obtained data was performed with the help of my master student Jiri TAPAL and were used for his master thesis: Tapal J., (2016): Optimalizace přenosového modelu *Babesia microti* [Optimization of *Babesia microti* transmission model. Mgr. Thesis, in Czech] – 59 p., Faculty of Science, University of South Bohemia, Ceske Budejovice, Czech Republic. Since the experiments listed in the Jiri's master thesis were designed to complete my doctoral research and were obtained under my supervision, I include them to my doctoral thesis. The experiments are marked by an asterisk (*).

Babesia microti: introduction to the problematics

Phylogenetically, *B. microti* is positioned on the basis of the order *Piroplasmida* and together with *B. microti*-like species (classified as *Theileria annae* or, the most recently, *B. vulpes* [115]) and *B. rhodaini* forms one clade/group [5, 12, 18-20]. The numerous analyses displayed the high distance and divergence of the *B. microti* clade from the other piroplasms [12], given by an early time point of its deviation. Interestingly, the *B. microti* clade comprises very high diversity of hosts species worldwide [29, 41, 115]. From the molecular point of view, *B. microti* is unique for its mitochondrial genome structure. Unlike other Apicomplexa, the mitochondrial genome of *B. microti* is circular [189, 190]. *B. microti* possesses the smallest genome among the Apicomplexan species. It consists in three chromosomes encoding ∼3500 polypeptides and sizes ∼6.5 Mbp (about 20% smaller compared to already sequenced genomes of other piroplasms) [189].

B. microti also differs from *Babesia sensu stricto* species ultrastructurally. The merozoites lack several organelles like a conoid, polar rings and microtubules. Intra-erythrocytic merozoites are shaped irregularly and possess numerous invaginations, pseudopods, twisting and coiling [40]. The cycle lacks the transovarial transmission and only transstadial transmission occurs in the *B. microti* cycle [94, 191-193]. The primary route of parasite transmission (Fig. 11) comprises larval ticks ingesting blood meal from an infected rodent reservoir. In the tick gut lumen, parasite undergoes a sexual reproduction by gametes fusion [46, 47]. Zygote then penetrates the peritrophic matrix [50] to invade gut epithelial cells [49]. Here it multiplies and resulting kinetes are believed to spread through tick tissues like nephrocytes and salivary glands [194]. After larvae feeding the molting to nymphs occurs. Sporogony in salivary glands starts after nymphs attachment to the consequent naïve host [52], in natural conditions represented by a rodent. *B. microti* fully matured sporozoites after 48h of tick engorgement [48, 52, 195, 196], ready to infect the vertebrate host and reproduce asexually in its red blood cells [197, 198].

The host range of *B. microti* natural reservoirs comprises particularly rodents [41]. A primary reservoir host in USA is represented by white-footed mouse (*Peromyscus leucopus*) [199], known to transmit the parasite into the ticks [200]. Field surveys estimate that ~40% of these mice are *B. microti* infected [199, 201] and the highest reservoir competence (average of 29.5% of ticks became infected) is attributed to them [41]. Pursuant to the seroprevalence the meadow voles (*Microtus pennsylvanicus*) has been established as another reservoir host [202], although less abundant. In USA, *B. microti* infections were reported also in the shrews [203], rabbits and chipmunks [199], racoons [204] and foxes [205]. Among these, the highest reservoir competence was defined for short-tailed shrews (*Blarina brevicauda*), and Eastern chipmunks (*Tamias striatus*) (21.9% and 17.6% infected ticks, respectively) [41]. In Europe, the other genera of mice (*Apodemus flavicollis, Apodemus sylvaticus, Mus* spp.), shrew (*Sorex araneus*), voles (*Microtus agrestis*, *Myodes*

glareolus) and raccoon dogs (*Nyctereutes procyonoides*) has been reported as *B. microti* natural hosts. *M. glareolus* is considered as a primary *B. microti* reservoir in the European countries [95, 97, 206-215]. *B. microti* is also distributed in various rodents in Asia, particularly in mice, voles, shrews, chipmunks or squirrels [216, 217].

The role of principal vector for *B. microti* in USA is attributed to *Ixodes scapularis* (= *Ixodes dammini*), known as deer or black-legged tick [218], where the questing nymphs are spreading the infection and are responsible for human babesiosis [199, 219]. The primary enzootic vector of *B. microti* in Europe is represented by *Ixodes trianguliceps*, a tick species that does not engorge on humans [103, 220]. This fact can explain the relatively few human diseases caused by *B. microti* which have been reported in Europe [58, 65, 82, 127, 129, 220]. The *I. ricinus*, the European sheep tick questing large animals and humans, also plays an important role in the transmission of the parasite among the field rodents [103]. It has been identified as *B. microti* competent vector [94] and recently, the *B. microti* infected *I. ricinus* ticks have been found throughout Europe [95-109]. The *B. microti* has been detected also in other tick species, e.g. *Dermacentor reticulatus* [221, 222].

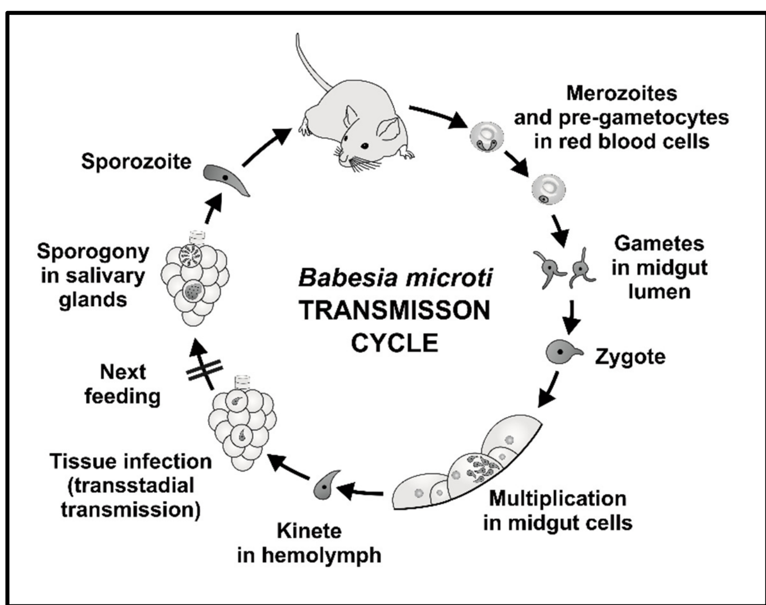

Figure 11. *B. microti* transmission cycle. Natural transmission cycle of *B. microti* involves rodents as the reservoir hosts and *Ixodes* ticks as vectors. Parasites in red blood cells of rodents are taken-up within the blood meal of larval stage of ticks, where they develop. During feeding of the next tick stage – nymphal – parasites are transmitted back into rodents. No transovarial (vertical) transmission occurs in *B. microti* transmission cycle. Figure was adapted from the illustration designed by M. Hajduskova (www.biographix.cz).

In laboratory conditions, *B. microti* is often used in studies evaluating effects of anti-parasitic compounds *in vivo* (e.g. [187, 223, 224]); almost exclusively infected laboratory mice are used in these studies. Beside the mouse, there are many susceptible laboratory animals like macaque (*Macaca mulatta*) [225], gerbils (*Meriones unguiculatus*) [94] and hamsters (*Phodopus sungorus* and

Mesocricetus auratus) [226, 227]. To date, just a few studies attempted to establish *B. microti* transmission model into laboratory conditions, using susceptible laboratory hosts. Using the hamsters, the transstadial parasite transmission from larvae to nymphs was documented for *I. ricinus* [192]. Analogous study using gerbils observed the same larvae to nymphs route of transmission and demonstrated also transstadial transmission from nymphs to adult stages [94]. The authors documented the prevalence 100% in molted nymphs (detected in pools of 2 nymphs), fed as larvae in acute phase of the infection. Adult ticks possessed prevalence 70 – 80 % (dependent on the strain) when acquired the parasite as nymphs. Yet, the parasite did not persist beyond more than one molting step [94]. In contrast, very low probability of transstadial transmission (both larvae-nymphs and nymphs-adults) was documented, using the primary European tick vector of *B. microti*, the *I. trianguliceps*, and voles as vertebrate hosts [209]. The transstadial transmission of *B. microti* in *I. dammini* (= *I. scapularis*) was observed for both routes (larvae-nymphs and nymphs-adults) but no data about the parasite prevalences are available [48, 52]. To date, the successful parasite transmission from ticks to gerbils or hamsters was reported only for the *I. scapularis* and *Ixodes pacificus* nymphal stage (infected at the larva stage) [191]. A very recent study reported the successful establishment of *B. microti* transmission model into laboratory conditions using laboratory mice and *Rhipicephalus haemahysaloides* as a tick vector; the parasite prevalence in molted nymphs and adults was 43.8% and 96.7%, respectively, and successful transmission from nymphs to mice was reported [193].

The experiments performed in order to establish *B. microti* laboratory model are described further (Chapter 1. Establishment of *B. microti* laboratory model). Subsequently, we used the implemented model for investigation of mutual immune interactions between the *B. microti* and *I. ricinus* (Chapter 2. Effect of tick immune genes on *B. microti* acquisition).

Pages 113-139 were removed because they contain unpublished data. If you are interested, please contact jalovecka@paru.cas.cz.

Discussion and future perspectives

The establishment of a complete laboratory cycle is a crucial step in investigation of all biological aspects of *Babesia* and babesiosis. The maintenance of *Babesia* parasites in laboratory conditions represents a challenging task due to the *Babesia* species diversity and wide spectrum of exploited hosts and vectors [41]. The difficulties are mainly coming from their vectorial transmission which implies mastering parasite maintenance in both vertebrate and invertebrate hosts of which tick represents the more challenging one. The primary vectors of *Babesia* are Ixodid (hard) ticks but the group comprises many species with different biological characteristics [87]. The ecology of *Babesia* includes a variety of wildlife animals as reservoir hosts but the *Babesia*-caused infections are frequently reported also from domestic animals and humans [41]

In the thesis, I focused on etiologic agents of human babesiosis, *B. divergens* and *B. microti.* The two species exhibit remarkably different biology and ecology [41]. Such diversity can be attributed to their evolutionary distance as *B. divergens* is a typical representative of *Babesia sensu stricto* lineage [12, 139-141] but *B. microti* is positioned on the basis of the order *Piroplasmida* and displays high distance and divergence from the other piroplasms [5, 12, 18-20]. *B. divergens* is naturally acquired by adult tick females during feeding on infected cattle and subsequently transmitted via eggs to larvae (Fig. 6) [57]. The transmission of *B. microti* occurs primarily via the larvae infestation and subsequent transmission to molted nymphs (Fig. 11) [192] but transstadial transmission from nymphs to adult stages was also demonstrated [94]. Such biological and ecological characteristics need to be taken into consideration when implementing the laboratory transmission model. In the thesis, I attempted to implement the laboratory model of both *B. divergens* and *B. microti* with the respect to natural developmental and transmission features.

Initially, I attempted to establish laboratory transmission cycle of *B. divergens* (see Part III.). The Mongolian gerbil represent the only known laboratory animal fully susceptible to *B. divergens* infection [170]. However, the infected gerbils exhibited considerable differences in parasite burden (Fig. 8) which resulted in difficulties to standardize conditions for parasite acquisition by ticks. It was previously reported that *B. divergens* growth in gerbils can be influenced by the size of parasite inoculum [151, 321]. Yet, in spite of the identical dosage, the gerbils displayed either resistance or development of a disease with disparity of maximal parasitemia. As was previously shown, the response of gerbils can vary despite the identical dosage [170]. Another factor potentially responsible for variations of parasite growth is an age of experimental animals [322]. Therefore, we attempted to infect animals of different age and sex but no standardization was reached (Fig. 8). It was previously documented that immature gerbils (younger than 1 month) are more resistant to the older animals (more than 2 months) [322] but still one third of our animals displayed the resistance

to *B. divergens* regardless the age. The likely explanation of such results can be attributed to the genetic heterogeneity of gerbils. In fact, the genetic heterogeneity might be assumed as a key factor causing the observed discrepancies, reflecting the uniform *B. microti* disease development in inbred laboratory mice (Fig. 12A). Gerbils does not belong to inbred laboratory animals although it was previously confirmed that laboratory gerbils display poor genetic diversity [323]. The disparity of parasite growth can be also given by utilization of gerbil itself since it does not represent the reservoir host for *B. divergens* [57].

Therefore, we optimized the technique of artificial tick feeding using the *in vitro* cultivated parasite (Fig. 9) since *B. divergens* can be long-term maintained in *in vitro* cultivated bovine erythrocytes [119, 165]. In our first experiments it was shown as a disadvantage since the long term *in vitro* culture lost the power to further develop in the tick (see the publication "Stimulation and quantification of *Babesia divergens* gametocytogenesis" [118]). These results implied the importance of gametocytes in the *B. divergens* lifecycle (and potentially in all vectorialy transmitted protozoan parasites, e.g. [173]). Gametocytes represent imperative developmental stages of *Babesia* lifecycle and determine the ability to infect the tick [3, 39, 46, 172]. Taking advantage of previously described and validated molecular markers for *B. divergens* gametocytes [142, 143] we verified the gametocytogenesis of the *in vitro* maintained parasite. The analysis showed that the parasite competence to produce gametocytes was lost during the long term *in vitro* cultivation [118]. It is necessary to concede that verification of the parasite infectivity to tick should have been assessed prior the first acquisition experiments, taking into account previously described and validated markers [142, 143]. We repeated the studies using a gametocytes-producing *B. divergens* strain and confirmed the parasite acquisition by ticks (Fig. 9) but the potential contamination of examined tissues by infected blood meal during the dissection process must be admitted. However, the transovarial transmission was not confirmed. We hypothesize that the high mortality of ticks fed on blood meal containing the parasites could be a sign of tick female infection since ticks fed on non-parasitized blood meal laid the standard amounts of eggs. Yet, several optimization procedures did not result in convincing result. Thus, the establishment of *B. divergens* complete cycle appeared as a challenging task. The issue is apparent also from the minority of reports presented in the literature. To date, only few studies described the successful parasite transovarial transmission [144-147] and there exists only one study documenting the *B. divergens* transmission to laboratory host via larvae feeding [144]. In the majority of these studies the tick females acquired the parasite during the engorgement on infected splenectomised calves [144] because the cattle represent the reservoir host for *B. divergens* [57]. Yet, experiments with the cows are beyond the scope of this work. Moreover, the studies reporting the parasite transmission in laboratory conditions did not subsequently continued in any experimental application of the established model like e.g. potential studies of mutual *Babesia*-tick interactions.

Since the importance of gametocytes for the *B. divergens* transmission model was implied, we further focused on studies considering the *B. divergens* sexual development and take an advantage of well-established *in vitro* culture of *B. divergens* and technique of gametocytes molecular detection. In general, the *Babesia* gamogony starts in the host bloodstream where the gametocytes occur [2, 44, 46, 53, 54, 118, 142, 324, 325]. The process of parasite sexual commitment is considered as stochastic and can be influenced by many factors. As documented for *Plasmodium*, the gametocytes determination is governed by the genetic and environmental factors [326]. Based on previously described and validated markers for *B. divergens* sexual commitment [142, 143], we established a technique for the quantification of *B. divergens* gametocytes. Using the technique, we monitored the kinetics of parasite sexual commitment upon the various stimuli (see the publication "Stimulation and quantification of *Babesia divergens* gametocytogenesis" [118]). The significant increase of gametocytemia was recorded during the continuous *in vitro* growth of the parasite. The phenomenon is probably related to accumulation of metabolic waste as no changes in gametocytemia were recorded after addition of lysed healthy erythrocytes. This hypothesis is supported by the fact that the maintenance of high parasitemia in cultures or regular medium replacements did not alter the parasite sexual commitment. The *B. divergens* sexual development was also stimulated by addition of widely used anti-babesial drugs, imidocarbe and atovaquone [55, 327]. The research of the *B. divergens* sexual commitment was intended to define the stimulants of gametocytes production, and thus increase the parasite infectivity to ticks. The observed stimuli of parasite sexual determination could serve as an improvement of parasite acquisition by ticks using the *in vitro* feeding technique. Nevertheless, the tick feeding on parasite *in vitro* culture represent a model divergent from natural *B. divergens* transmission. Therefore, we decided to further focus on *B. microti* (see Part IV.). The biology of this species allowed us to use laboratory mice, and thus be more respective to transmission cycle of *B. microti* in natural conditions. The *B. microti* offers a uniform course of infection in the laboratory mice (Fig. 12A), and thus a better standardization of the transmission model. With the respect to reported variations in *B. divergens* growth in gerbils (Fig. 8), the consistent course of *B. microti* infection might be related to usage of inbred laboratory animal. The BALB/c mice belong to the most widely used inbred strain of laboratory mice [328] which contradicts with utilization of gerbil as an laboratory animal, although the gerbil genetic heterogeneity was previously reported as low [323]. Another potential explanation of observed discrepancy correlates with natural hosts of both parasite species since gerbil does not represent the reservoir host for *B. divergens* [57] but mouse as a rodent does for *B. microti* [41, 199, 202].

The *B. microti* growth curve was categorized into three phases (Fig. 12A) consisting of the rapid parasite multiplication (acute phase), gradual decrease of parasitemia (decline phase) and low parasitemia persisting for a long period (latent phase). The parasite acquisition by larval ticks during acute phase appeared as the most effective for subsequent transstadial transmission to molted

nymph. Also, ticks fed on hosts in acute phase of the infection displayed the highest infectivity to naïve host (Fig. 13) but this is most likely given by the high parasite prevalence in the tick pool fed on laboratory mice. These results fully correspond with transmission studies exploiting the gerbil hosts [94] and could be related to parasite sexual commitment. The identification of parasite gametocytemia would have been highly desirable but no markers for *B. microti* gametocytes have been described and validated so far. With the respect to observed stimulation of gametocytemia during the rapidly increasing parasitemia of *B. divergens* [118], we assume the same phenomenon for the *B. microti* intra-erythrocytic multiplication. We hypothesize that the rapid expansion of *B. microti* during the acute phase of the mice infection is probably related to the increase of sexual commitment, and thus higher parasite infectivity to ticks. This most likely explains the discrepancy in parasite prevalence in ticks fed in acute phase compared to ticks engorged during decline and latent phase.

Interestingly, we documented low parasite prevalence in ticks which acquired the parasite during the latent phase of the host infection. Our results fully correspond with the studies reporting lack of parasite infectivity during the chronic infection [94, 209]. Yet, it raises question about the parasite maintenance in natural conditions since the *B. microti* parasitemia is believed not to exceed 1% in wildlife rodents [329]. It also explains low prevalence of *B. microti* reported form the field ticks (~ 0.5-5%) [95-109]. This issue could be related to observed parasite seasonality (Tab. 3). Reflecting numerous repeated experiments, we observed a fluctuation of documented parasite prevalence in ticks which differed in relation to season periods, despite uniform experimental conditions and ticks maintained in laboratory conditions. Seasonal variations of *Babesia* parasite have been previously documented in ticks as well as field rodents [199, 238], showing the increased parasite prevalence during spring months. The observed seasonal disparity might be also related to the spring peak of *I. ricinus* questing activity [87, 239] since the seasonal distribution was reported for other tick-transmitted pathogens [240]. In spite of accordance of our results with these observations, we have to admit that more additional experiments would be required to postulate the definitive conclusion.

The proposed parasite seasonality is highly unfeasible for any laboratory experiments, although it may reflect the parasite biological characteristics. Thus, we intended to optimize the parasite transmission model by changing of tick breeding temperatures, as the positive temperature modification of tick susceptibility to *Babesia* parasites was previously reported [241]. We documented the remarkable increase of parasite prevalence in tick nymphs by incubation of the larvae tick stages in higher temperatures (30 and 35°C) prior the parasite acquisition via feeding on infected host (Fig. 14). It corresponds to mammalian host temperature (~37°C), suggesting that the temperature increase may be the key point of parasite effective acquisition during the tick feeding. Yet, it is imperative to admit that larvae exposure to high temperatures might represent a stress

condition for the tick. Therefore, it can potentially result in modification of tick innate protection to the parasite, and thus facilitate the parasite acquisition. The larvae exposure to high temperature also does not reflect the parasite ecology and biology since lower temperatures are preferred for the tick development [243, 244]. Thus, the optimized laboratory model might be divergent from *B. microti* transmission in natural conditions. However, the established model offers a great platform for studying the parasite biology since it allows to get higher average parasite prevalence in experimental ticks compared to usual prevalence in field ticks [95-109].

It was previously reported that *B. microti* invades several tick tissues during the acquisition process [194] and parasite survives the tick ecdysis in the form of dormant sporoblast in tick salivary glands as proven in studies exploiting *I. scapularis* (= *I. dammini*) [48, 52]. In accordance, we documented the parasite persistence in tick salivary glands after the tick molting (Tab. 4). The sporozoites differentiation begins with the blood uptake and is assumed to proceed at least 48h [48, 52, 195, 196]. It implies that sporozoites can occur in the tick saliva after 2 days of tick engorgement, and thus the parasite transmission into the host bloodstream does not occur at the first days of the tick bite. Our *B. microti* model is characterized by parasite transmission into the host bloodstream exclusively via ticks fed to full repletion (Fig. 17). The removal of ticks during the first 48h of tick bite effectively prevented transmission of sporozoites able to induce the host fulminating infection. In a broader perspective, such transmission dynamics is a crucial fact for the babesiosis prevention strategy since the early tick elimination could prevent the disease development.

However, the parasite persistence in acinar cells of molted nymphs (Tab. 4) limits the potential investigation of interactions at *Babesia*-tick interface using the specific gene silencing because the technique is not yet feasible in tick larvae. Since the tick defensive mechanisms exhibit an efficacy particularly in the haemolymph [56-60], the potential anti-babesial mechanisms occur primarily during the parasite acquisition during the tick engorgement. Therefore, the established transmission model possesses a limitation for the research of mutual *Babesia*-tick interactions. In order to evade this incompatibility, we established *B. microti* acquisition model exploiting the infestation of tick nymphs (Fig. 3). Although we must admit that parasite acquisition by tick nymph tick is not the primary transmission route of *B. microti*, the field studies confirm its frequent occurrence (reviewed in [41]). The parasite invasion into acinar cells appears prior the tick full engorgement (Fig. 18 and 19) and reflects the parasite gamogony and subsequent kinetes spread in the tick body. Since the parasite dissemination is mediated via kinetes migration through the haemolymph, we can assume that *Babesia* may interact with tick immune mechanisms. Ticks exhibit the powerful anti-microbial response (reviewed in [1, 284]) which includes the immune signalling pathways and subsequent putative production of downstream effector molecules. Since no knowledge is available about regulation of protozoan infection in ticks, we evaluated the three principal immune signalling

pathways, the Toll pathway, the Imd pathway and the Jak/Stat pathway [262]. Using the technique of specific gene silencing we demonstrated the potential role of Toll immune pathway and plausible cooperation of Toll and Imd signalling (Fig. 22A, B, C). Based on these results we hypothesize that tick anti-babesial response is orchestrated by divergent signalling pathways than response to *A. phagocytophilum*, which appears to be primarily regulated by Jak/Stat pathway [123, 313, 314]. Moreover, the suggested role of Toll and Imd signalling is corresponding to mosquito control of *Plasmodium* invasion [277].

In the mosquitos, both Toll and Imd pathways were proven to regulate the mosquito anti-plasmodial response via stimulation of TEP1, the complement C3-like protein from the group of thioester-containg proteins [277]. Identification and description the tick thioester-containg proteins [120] thus imply the potential anti-babesial effect of these molecules. This hypothesis is supported by our results (Fig. 22D and 24) which indicate a commitment of TEP (thioester containing protein, homologue of insect TEP1) and MCR (macroglobulin related proteins 1+2) molecules in *B. microti* acquisition by ticks. Reflecting the previously demonstrated anti-bacterial activity of tick thioester-containing proteins [120, 121, 124], we can hypothesize that tick anti-microbial and anti-parasitic response might be regulated by different representatives of tick thioester-containg proteins. Moreover, the suggested efficacy of TEP molecule in tick anti-babesial response indicates that mechanisms controlling the protozoan invasion of mosquitos and ticks might be higly similar, despite the different biology of both vectors. Based on our contemporary data we cannot confirm the connection both NF-κB-related pathways and the tick thioester-containing proteins, like it was defined for mosquito. Since these experiments are highly desirable, we currently continue in this direction. To conclude, the research targeted to elucidation of tick anti-babesial response provides a view into mechanisms of parasite vectorial transmission, and thus potentially could help to prevent *Babesia* transmission by ticks. In a broader perspective, the cross-talk between the parasites and vector occupies the pathogen strategies often relying on the passive or active host participation [330]. The identification of such genes and molecules regulating the parasite burden in the vector can further shape the vectorial capacity of pathogen transmission.

To support the research in the field of *Babesia*-tick interactions, we currently focus on the transcriptomic characterization the tick and babesial genes activated during the parasite transmission to the vector and persistence in its salivary glands. We prepared the RNA extracts of salivary glands at different time points of tick feeding (0h, 24h and 72h) using highly infected tick nymphs (prevalence >50%); uninfected salivary glands were used as a control in the same manner. The transcriptome analysis was performed by GenXPro (http://tools.genxpro.net/) and we have recently received the transcriptomic data. Data are currently analyzed in collaboration with other

laboratories in scope of the project ANTIDotE: anti-tick vaccines to prevent tick-borne diseases in Europe [331].

The platform of established laboratory model(s) exhibits a great potential for many research ways. Exploiting the technique of *Babesia* molecular detection used in our research, we participated on the research evaluating the microscopy and PCR as diagnostics tool for *Babesia* (see publication "Validate or falsify: Lessons learned from a microscopy method claimed to be useful for detecting *Borrelia* and *Babesia* organisms in human blood" [117]). The other research direction benefits from well-established *in vitro* maintenance of *B. divergens* which offers a great model for testing innovative anti-babesial therapeutic trends. We focused on the proteasome inhibitors which are currently reported as a powerful strategy for treatment of parasitic diseases [44-49]. Up to now, we validated the *Babesia* proteasome as a valuable drug target and verified the strong anti-babesial effect of commercially available compounds (see the manuscript in preparation "Validation of *Babesia* proteasome as a drug target"). We intend to carry on with design of new compounds targeting the *Babesia* proteasome. Subsequently, we aim to verify *Babesia* burden in host bloodstream as well as within the tick organs, and thus exploit the transmission and acquisition models of *B. microti* in a greater extent.

Thesis summary

The thesis provides an insight to biology of *Babesia microti* and *Babesia divergens*, agents of human and bovine babesiosis with increasing incidence worldwide. In the thesis we provide a comprehensive literature review and present the results obtained during the doctoral research. Thesis includes previously published research and unpublished data, which are thoroughly described and discussed to point out their interest for the scientific community.

PART I. GENERAL INTRODUCTION

The first part of the thesis provides a comprehensive review on current knowledge of the problematics. *Babesia* is a genus of many species, taxonomically placed into the order *Piroplasmida* together with *Theileria* and *Cytauxzoon* [12]. Based on multi-gene analyses, piroplasms are closely related to *Plasmodium*, an agent of malaria disease [9-12]. As other apicomplexan organisms, the parasite cell is equipped by Apical complex. This unique collection of organelles mediates the penetration of the parasite into the host cell, and thus is found in all invasive stages [34].

Babesia developmental cycle is a series of complex events exploiting vertebrate organisms as hosts and ticks as vectors. Current research pays a great attention to parasite multiplication in the host cells. Yet, the parasite development in the tick organism is poorly described. This is given by partial lifecycle descriptions scattered in many publications, non-uniformity of individual stages nomenclature and recent redescription of many species [21, 115, 116]. Therefore, we decided to analyse the lifecycle of piroplasms in the manuscript in preparation "Lifecycle of piroplasms: comprehensive analysis". Here we provide a detailed view into developmental events of representative piroplasms species – *Theileria*, *Cytauxzoon* and *Babesia* – in context of the latest knowledge about the taxonomy, unique features in their life cycle and novel nomenclature.

Babesia parasites are cosmopolitan parasites with extremely wide host range which includes hundreds of mammal hosts. Currently, *Babesia* is considered as the second most common blood parasite worldwide [13]. Babesiosis is an emerging disease of economical, veterinary and medical importance. It affects livestock, wildlife and companion animals [4, 7, 60-62], and humans [13, 56, 58, 59]. Bovine babesiosis is recognized for its major impact on farm animal health and associated with enormous economic losses the livestock industry [4, 57, 62]. Human babesiosis is reported as current major blood transfusion threat [6, 41, 58, 66] and possess a significant risk particularly to immunosuppressed patients [68, 73-78]. The disease is transmitted by ticks and to date the ticks of the family *Ixodidae* have been reported as the exclusive vectors of *Babesia* parasites [41]. In the Europe, *Babesia* species are primarily transmitted by *Ixodes ricinus* [87] and incidence of bovine and human diseases is attributed to distribution and density of *I. ricinus* [6, 13, 55, 57, 62, 65].

Since tick serves as a host and vector for many pathogens including *Babesia* parasites, it possesses innate immune mechanism interacting with the transmitted microbes. In order to summarize these interaction, we published the review "Interaction of the tick immune system with transmitted pathogens" [1]. In the review we provided a comprehensive analysis of the tick antimicrobial immune mechanisms mediating cross-talk with three the most intensively studied tick-transmitted pathogens – *Borrelia*, *Anaplasma* and *Babesia* – in context of their natural development in the internal organs of vector ticks. In this publication, I participated on summarizing knowledge about tick molecular mechanisms interacting with-*Babesia* parasites.

PART II. SUMMARY OF RESEARCH METHODS

The second part summarizes methods used in the thesis in order to establish *Babesia* laboratory transmission model and study the molecular interactions at the tick-*Babesia* interface. The chapter describes wide spectrum of laboratory techniques and graphically depict the schemes of transmission experiments. We introduce here the novel methods in *Babesia* research like utility of fluorescence *in situ hybridization* for the visualization of parasite development inside the tick salivary glands. The chapter includes one methodical publication "Validate or falsify: Lessons learned from a microscopy method claimed to be useful for detecting *Borrelia* and *Babesia* organisms in human blood" [117]. The research presented in publication was conducted in cooperation with Department of Bacteriology and Immunology, Norwegian Institute of Public Health, Oslo, Norway and I participated on *Babesia* spp. detection in the human blood samples. In the publication, the microscopy as diagnostics tool for *Babesia* (and *Borrelia*) was evaluated and compared with the molecular diagnostic techniques. The publication confirmed that microscopy could falsify the diagnosis due to misinterpretation of staining artefacts. Thus, the molecular techniques should be used to confirm a definitive diagnosis of human babesiosis but rigorous precautions are required to avoid false-negative or false-positive results. The incidence of human babesiosis is recently reported in many countries (reviewed e.g. in [6, 41]). Therefore, the reliable diagnostician techniques are required for diagnostics accuracy and further effective treatment.

PART III. Establishment of *Babesia divergens* laboratory model and its experimental application

The third part of the thesis provides a comprehensive literature review about *B. divergens*, a European agent of human and bovine babesiosis, and summarize experiments performed in order to introduce complete laboratory model of *B. divergens. B. divergens* belongs to the *Babesia sensu stricto* group which is characterized by transovarial transmission in ticks and multiplication exclusively in erythrocytes of the vertebrate host [5, 12, 18-20]. A successful tick infection is dependent on development of parasite gametocytes, first sexual stages occurring in the blood stream of the vertebrate host. Since *B. divergens* is maintained *in vitro* and undergoes repeated passages, these sexual stages can be reduced or dispatched. Based on previously described and

validated markers of *B. divergens* gametocytes [142, 143], we introduced the technique of gametocytes quantification. The results were published in the publication ""Stimulation and quantification of *Babesia divergens* gametocytogenesis" [118]. In this publication I performed all the described experiments using the quantification technique and wrote the manuscript. I also acted as a corresponding author of the publication. The research provides an insight into *B. divergens* sexual development. We monitored parasite sexual commitment kinetics upon the various stimuli and identified incentives significantly increasing the gametocytemia. The results presented in the publication has the potential to facilitate further research in the field of *Babesia*-tick interactions.

The chapter also includes the unpublished data obtained during the establishment of *B. divergens* transmission model in the laboratory conditions. Using the primary vector of *B. divergens*, the tick *I. ricinus*, we attempted to introduce the model using *in vivo* tick feeding on infected gerbils. The gerbils exhibited considerable variations in parasite growth in the host bloodstream which makes difficult to standardize conditions for parasite acquisition by ticks. Thus, we decided to replace the laboratory animals by artificial feeding technique [126]. We documented a successful parasite acquisition by female ticks but the transmission cycle was not completed. Due to observed difficulties during the establishment of *B. divergens* laboratory model we decided to attempt another parasite species. The *B. microti* represents more convenient model organism due to feasible *in vivo* maintenance in laboratory mice. The results concerning the *B. microti* are summarized in the fourth part of the thesis.

Yet, the establishment of *B. divergens in vitro* culture offered new research ways. The long term *in vitro* maintenance of *B. divergens* represents a great model for testing of innovative anti-babesial therapeutic trends. The development of new anti-babesial drugs is highly desirable due to major side effects of current babesiosis therapy [73, 178]. Human babesiosis is usually treated by combination of antimalarial and antibacterial drugs [6] and possess numerous side effects and even drug failure related to increase of resistant parasites [73, 178, 179]. Using established *B. divergens in vitro* cultures we conducted the primary screening of selected commercially available compounds and analysed their anti-babesial effect. The proteasome inhibitors are currently in the centre of high interest due to great anti-parasitic efficacy. Proteasome is multi-component protein complex regulating many cellular processes [188] and recent findings reported proteasome inhibitors as powerful strategy for treatment of parasitic diseases [180-185]. Therefore, we verified the potential of proteasome inhibitors to serve as anti-babesial therapeutic agents. Results of this research are summarized in the manuscript in preparation ""Validation of *Babesia* proteasome as a drug target". I was involved in all experiments described in the manuscript including their

analysis and interpretation. I also participated in the manuscript writing. The manuscript in preparation validates proteasome inhibitors as potential viable anti-babesial drugs.

PART IV. Establishment of *Babesia microti* laboratory model and its experimental application

The fourth part of the thesis provides a comprehensive literature summary about *B. microti*, an agent of human babesiosis, and describes unpublished research concerning the establishment of *B. microti* laboratory model(s). *B. microti* is phylogenetically placed on the basis of the order *Piroplasmida* [12]. Compared to other apicomplexan parasites, *B. microti* exhibits a remarkable differences like the very small genome [189] or reduced apical complex [40]. In contrast to species of *Babesia sensu stricto* lineage, the *B. microti* cycle lacks the transovarial transmission. The parasite possess only transstadial transmission, usually from tick larvae to molted nymphs [94, 191-193]. As reservoir hosts of *B. microti* serve rodents [41]. This represents a facilitation for establishment of parasite laboratory cycle since parasite is feasible maintained in common laboratory mice.

In order to introduce the *B. microti* laboratory model, we adapted the parasite infection in BALB/c laboratory mice and monitored the standard course of infection. The mice infection was categorized into three different phases describing the rapid increase of parasitemia (acute phase), gradual decrease of infected erythrocytes (decline phase) and long-term parasite persistence in very low parasitemia levels (latent phase). The maximal parasitemia was observed 6th day post infection, reaching 55 ± 3.9%. To respect parasite natural transmission, we established a model using pathogen-free larvae fed on infected laboratory mice. Subsequently, we evaluated the *B. microti* prevalence in molted nymphs and monitor parasite transmission to naïve mice. We documented high prevalence in ticks fed in acute phase (89%) compared to decline and latent phases (2.5% and 7.6%, respectively). Moreover, the pool of 10 ticks fed in the acute phase transmitted the parasite into all experimental naïve mice. Thus, the mice in acute phase of the infection were used in subsequent experiments. Despite the successfully established *B. microti* laboratory model, we documented the seasonal variances influencing the model achievement. In many attempts we observed high parasite prevalence in ticks during spring months compared to very low prevalence obtained in autumn and winter season. Using larvae incubation in high temperatures (35°C) we optimized the established model and avoid the seasonal variances. Employing the optimized model, we assessed the parasite development in various tick tissues. We documented the *B. microti* persistence exclusively in salivary glands during the tick nymph progeny. The parasite presence in tick salivary glands was confirmed by parasite sporozoites visualization using the technique of fluorescence *in situ* hybridization. Moreover, we observed the host infection with *B. microti* exclusively after tick full engorgement. This suggests that development of matured sporozoites – able to infect naïve host – lasts 48 hours after beginning of tick engorgement. Overall, we successfully established and optimized the *B. microti* transmission model. Yet, the parasite persistence in the

salivary glands of molted nymphs limits an investigation of tick innate immunity. The tick anti-microbial molecules possess an efficacy during the pathogen invasion particularly in the haemolymph [120, 121, 123, 124, 284]. Thus, the parasite biology in the established model eliminates the research of *Babesia*-tick interaction using the specific gene silencing since the technique is not feasible in tick larvae yet.

To avoid this complication, we implemented acquisition model of *B. microti*. The acquisition model is based on the *I. ricinus* pathogen-free nymph engorged on infected laboratory mice. The parasite acquisition by ticks is a complex issue comprising parasite sexual development in the tick midgut and subsequent invasion of salivary glands via haemolymph [46, 51, 52]. Using acquisition model, we assessed the chronology of *B. microti* dissemination into the tick salivary glands and attempted to visualize parasite stages in acinar cells. Based on several repetitions, we confirmed the uniformity and seasonal independence compared to the previously established transmission cycle. Thus, the acquisition model is further used in the research of mutual interaction between *Babesia* parasite and tick vector.

Ticks possess powerful anti-microbial mechanisms regulating pathogen invasion. The foreign organisms are recognized by the immune signalling pathways which further amplify the immune response by production of effector antimicrobial molecules. This chapter of the thesis describes the three most characterized tick signalling pathways orchestrating the anti-microbial response and further evaluates their role in acquisition of *Babesia* parasites. Our results pointed out to the importance two NF-κB-related signalling pathways – the Toll innate immune signalling pathway and the Imd (immune deficiency) pathway – in regulation of parasite acquisition. We also implied the anti-babesial effect of thioester-containing proteins (TEPs). TEPs are the homologs of human complement factors [120] and were previously identified to mediate the *I. ricinus* anti-microbial response [120, 121, 124]. Moreover, the TEPs were determined as downstream effector molecules of Toll signalling pathway in anti-malarial response of mosquito vector [277, 279]. We documented the commitment of two tick TEPs – complement-like protein TEP and macroglobulin related proteins (MCRs) – in the regulation of *B. microti* acquisition by ticks. Although we have not yet confirmed the connection of NF-κB-related pathways and tick TEPs (the experiments are ongoing), we can conclude that tick actively regulates the *B. microti* invasion via innate immune signalling and anti-microbial mechanisms of tick complement system.

Concluding remarks

The thesis provides a comprehensive review of my doctoral research. We introduce here fully optimized quantification model of *Babesia* parasite which is further used to several experimental applications. We investigate the parasite development and emphasized the *B. divergens* sexual commitment. We identified the tick signalling and immune mechanism regulating the *B. microti* invasion. We proposed a new direction of anti-babesial therapy by validation of the *B. divergens* and *B. microti* proteasome as a drug target. Overall, the research presented in the thesis extends the current knowledge of the *Babesia* parasite biology.

Résumé de la thèse [in French]

La thèse donne un aperçu de la biologie de *Babesia microti* et de *Babesia divergens*, agents de la babésiose humaine et bovine d'incidence croissante dans le monde entier. Dans la thèse, nous apportons une revue exhaustive de la littérature et présentons les résultats acquis au cours de la période doctorale. La thèse inclut des résultats publiés ou non, qui sont décrits en détails et discutés pour mettre en avant leur intérêt pour la communauté scientifique.

Partie I. Introduction générale

Cette première partie de la thèse fournit une revue détaillée et aussi exhaustive que possible des connaissances générales liées à la problématique. *Babesia* est un genre comportant une centaine d'espèces, placé taxonomiquement dans l'ordre des *Piroplasmida*, avec *Theileria* et *Cytauxzoon* [12]. Sur la base d'analyses phylogénétiques récentes et multigéniques, les piroplasmes ont été démontrés comme étroitement liés à *Plasmodium,* agent responsable de la malaria [9-12]. *Babesia* possède un complexe apical, caractéristique commune aux membres du phylum des Apicomplexes. Cet assortiment unique d'organelles assure la pénétration du parasite dans la cellule hôte, et est donc présent chez tous les stades invasifs [34].

Le cycle de développement de *Babesia* comporte une série d'évènements complexes exploitant les organismes vertébrés comme hôtes et les tiques comme vecteurs. Les recherches actuelles sont centrées presque exclusivement sur la multiplication du parasite dans les cellules de l'hôte vertébré. Le développement du parasite chez la tique est souvent négligé et décrit de façon superficielle et partielle. La difficulté provient de données descriptives partielles éparpillées dans de nombreuses publications, la non-uniformité de la nomenclature utilisée pour les différents stades et la re-description récente de nombreuses espèces [21, 115, 116]. Nous avons donc décidé d'apporter une vision la plus complète possible du cycle des piroplasmes chez la tique dans le manuscrit en préparation “Lifecycle of piroplasms: comprehensive analysis”. Dans cette revue de synthèse, nous apportons une vision détaillée des évènements du développement chez le vecteur des espèces représentatives des trois genres de piroplasmes – *Theileria*, *Cytauxzoon* et *Babesia* - dans le contexte des dernières connaissances sur leur taxonomie, des caractères uniques de meur cycle et des nouvelles nomenclatures.

Les parasites du genre *Babesia* sont très cosmopolites, avec une très large gamme d'hôtes qui inclut des centaines d'hôtes Mammifères. Actuellement, *Babesia* est considéré comme le second parasite sanguin le plus commun au monde [13]. La babésiose est une maladie émergente d'importance économique vétérinaire et médicale. Elle affecte le bétail, la faune sauvage, les animaux de compagnie [4, 7, 60-62] et l'homme ne fait pas exception [13, 56, 58, 59]. La babésiose

bovine est reconnue pour son impact majeur sur la santé des animaux de rente et associée à des pertes économiques énormes dans l'industrie du bétail [4, 57, 62]. La babésiose humaine est rapportée comme étant une menace majeure pour les transfusions sanguines [6, 41, 58, 66] et pose un risque significatif en particulier chez les patients immuno-déprimés [68, 73-78]. Cette maladie est transmise par les tiques et actuellement seules les tiques de la famille des *Ixodidae* ont été identifiées comme vecteurs des parasites du genre *Babesia* [41]. En Europe, les différentes espèces de *Babesia* sont principalement transmises par *Ixodes ricinus* [87], et l'incidence des la babésiose bovine et humaine est attribuée à la distribution et à la densité d'*I. ricinus* [6, 13, 55, 57, 62, 65].

Puisque la tique sert de vecteur à de nombreux pathogènes incluant *Babesia*, elle possède un mécanisme d'immunité inné interagissant avec les microbes transmis. Nous avons publié un résumé des informations concernant ces interactions dans une revue de synthèse "Interaction of the tick immune system with transmitted pathogens" [1, 13]. Dans cette synthèse, nous apportons une analyse des mécanismes immunitaires antimicrobiens qui régissent les interactions avec les trois pathogènes transmis par les tiques les plus étudiés – *Borrelia*, *Anaplasma* et *Babesia* – dans le contexte de leur développement naturel dans les organes internes des tiques vectrices. Dans cette publication, j'ai réalisé la partie concernant les connaissances sur les interactions immunitaires moléculaires tique - *Babesia*.

Partie II. Résumé des méthodologies

La seconde partie résume les méthodes utilisées dans la thèse dans le but d'établir un modèle de transmission de *Babesia* et d'étudier les interactions moléculaires à l'interface tique-*Babesia*. Ce chapitre décrit le large panel de méthodes de laboratoire employées et précise les schémas des expériences de transmission. Nous abordons dans ce chapitre des méthodes nouvelles dans les recherches sur *Babesia*, telles que le le FISH (fluorescence *in situ hybridization*) pour la visualisation du développement parasitaire dans les glandes salivaires. Ce chapitre inclut une publication méthodologique "Validate or falsify : Lessons learned from a microscopy method claimed to be useful for detecting *Borrelia* and *Babesia* organisms in human blood" [117]. Ces travaux ont été menés en coopération avec le "Department of Bacteriology and Immunology, Norwegian Institute of Public Health, Oslo, Norway" et j'ai participé à la détection de *Babesia* spp. dans les échantillons de sang humain. Dans cette publication, la microscopie en tant qu'outil diagnostic pour *Babesia* (et *Borrelia*) a été évaluée et comparée avec des techniques moléculaires. La publication confirme que la microscopie pouvait rendre un diagnostic erroné du à la mauvaise interprétation d'artéfacts de coloration. Il apparaît donc que des techniques moléculaires devraient être utilisées pour confirmer le diagnostic de babésiose humaine mais des précautions rigoureuses sont nécessaires pour éviter les faux positifs et les faux négatifs. L'incidence de la babésiose humaine a été récemment rapportée comme en augmentation, et des cas cliniques sont déclarés dans de nombreux pays [6,

41]. Des techniques de diagnostic fiables sont donc requises pour une bonne précision et un traitement efficace.

<u>Partie III. Mise en place au laboratoire du cycle de développement de *Babesia divergens* et ses applications expérimentales</u>

La troisième partie de la thèse fournit une revue complète de la littérature sur *B. divergens*, l'agent européen de la babésiose bovine et humaine, et résume les expériences réalisées pour mettre en place un modèle complet de transmission de *B. divergens* au laboratoire. *B. divergens* appartient au groupe des *Babesia sensu stricto*, groupe caractérisé par une transmission transovarienne chez le vecteur et une multiplication cellulaire limitée aux érythrocytes de l'hôte vertébré [5, 12, 18-20]. L'infection de la tique lors du repas de sang infecté est dépendante de la formation par le parasite des gamétocytes, premier stade de la phase sexuée du parasite qui apparaît dans le sang de l'hôte vertébré. Leur détection et leur quantification dans le sang permettent de déterminer la capacité du parasite à être transmis au vecteur. Sur la base de marqueurs décrits préalablement comme spécifiques des stades gamétocytes par rapport aux stades asexués [142, 143], nous avons mis en place une technique de quantification de ces stades. Les résultats ont été publiés dans l'article "Stimulation and quantification of *Babesia divergens* gametocytogenesis" [118]. Pour ce travail, j'ai réalisé l'ensemble des expérimentations, j'ai rédigé mon premier manuscrit et j'ai assuré le rôle de "corresponding author". Les travaux ainsi réalisés ont permis d'apporter des connaissances sur le développement des stades sexués de *B. divergens*. J'ai ainsi montré que le maintien à long terme en culture in vitro provoquait la perte de la capacité du parasite à produire ces stades, pourtant essentiels à sa transmission. J'ai analysé l'influence de divers stimuli sur la capacité du parasite à produire ces stades et ainsi pu identifier des conditions favorables à leur production. Les résultats présentés dans cette publication devraient permettre de faciliter des travaux futurs dans le domaine des interactions *Babesia* – tique.

Ce chapitre inclut également des données non publiées obtenues lors des tentatives de mise en place des étapes de transmission du parasite au vecteur en conditions de la boratoire. En utilisant le vecteur de *B. divergens*, la tique *I. ricinus*, nous avons tenté d'infecter des tiques *in vivo* à partir de gerbilles infectées. Mais la grande variabilité de croissance du parasite dans le sang de cet animal nous a contraint à remplacer le modèle d'infection via l'animal par une technique de gorgement artificiel [126]. L'acquisition du parasite par les tiques femelles a été démontrée, mais le cycle de transmission complet n'a pas été obtenu. En raison des difficultés rencontrées avec ce parasite, nous avons décidé de tester ces méthodologies sur une autre espèce parasitaire. *B. microti* représente un organisme modèle plus pratique en raison de la possibilité de le maintenir in vivo en souris de laboratoire. Les résultats concernant B. *microti* sont détaillés dans la quatrième partie de la thèse.

Cependant, la mise en place la culture *in vitro* a offert des voies nouvelles de recherche. Le maintien in vitro à long terme de *B. divergens* représente un atout pour tester de nouvelles voies thérapeutiques anti-*Babesia*. La recherche de nouvelles drogues anti-*Babesia* est importante. En effet, le traitement de la babésiose humaine comporte habituellement une combinaison de molécules anti-malaria et anti-bactériennes [6] qui ont de nombreux effets secondaires et avec de nombreux échecs dus à l'augmentation de parasites résistants [73, 178, 179]. En utilisant les cultures *in vitro* de *B. divergens,* nous avons réalisé un premier screening de molécules disponibles commercialement et analysé leur effet anti-*Babesia*. Le protéasome est un complexe enzymatique multi-protéique qui régule de nombreux processus cellulaires [188] et des travaux récents ont montré que les inhibiteurs de protéasome représentent des candidats prometteurs pour le traitement des maladies parasitaires [180-185]. Nous avons donc vérifié l'intérêt de ces inhibiteurs comme agents thérapeutiques contre *Babesia*. Les résultats de ces travaux sont compilés dans le manuscrit en préparation "Validation of *Babesia* proteasome as a drug target". J'ai réalisé l'ensemble des expérimentations décrites dans le manuscrit, ainsi que l'analyse et l'interprétation des résultats. J'ai également participé à la rédaction du manuscrit. Les résultats valident les inhibiteurs de protéasome en tant que molécules potentielles anti-*Babesia*.

Partie IV. Mise en place au laboratoire du cycle de développement de *Babesia microti* et ses applications expérimentales

La quatrième partie de la thèse apporte un résumé de la littérature sur *B. microti*, un des agents de la babésiose humaine, et décrit des travaux non publiés correspondant à la mise en place d'un modèle de laboratoire de transmission de *B. microti. B. microti* est phylogénétiquement placé à la base de l'ordre des *Piroplasmida* [12]. *B. microti* se démarque des autres parasites apicomplexes par plusieurs caractéristiques fondamentales telles que le toute petite taille de son génome [189] ou à son complexe apical réduit [40]. Contrairement aux espèces de la lignée des *Babesia sensu stricto*, le cycle de *B. microti* ne comporte pas de transmission trans-ovarienne. En conditions naturelles, le parasite ne se transmet que de façon transstadiale, habituellement de larves à nymphes [94, 191-193]. Les rongeurs servent de réservoirs à *B. microti* [41]. C'est un simplification important pour établir au laboratoire le cycle du parasite car celui-ci est maintenu aisément chez la souris de laboratoire.

Pour mettre en place le modèle de transmission de *B. microti*, nous avons réalisé l'infection parasitaire dans des souris BALB/c de laboratoire et suivi l'évolution normale de l'infection. Trois phases différentes d'infection ont été séparées : l'augmentation rapide de la parasitémie (phase aigüe), le déclin progressif de la parasitémie (phase de déclin) et la persistence à long terme d'une parasitémie très basse (phase latente). La parasitémie maximale a été observée 6 jours après infection, avec des valeurs atteignant 55 ± 3.9%. Pour respecter les modalités de transmission

naturelle, le modèle de transmission a été mis en place en utilisant des larves indemnes de pathogènes que nous avons gorgées sur des souris infectées. Nous avons ensuite évalué la prévalence de *B. microti* chez les nymphes et contrôlé la transmission du parasite de ces nymphes vers des souris naïves. La prévalence d'infection est très élevée chez les nymphes issues de larves gorgées au cours de la phase aigüe (89%) et faible chez les nymphes issues de larves gorgées au cour de la phase de déclin ou latente (respectivement 2.5% et 7.6%,). De plus, les lots de 10 tiques infectées au cours de la phase aigüe ont transmis expérimentalement le parasite à toutes les souris naïves. La phase aigüe de l'infection a donc été sélectionnée pour la suite des expériences de transmission. Cependant, des variations que nous avons qualifiées de saisonnières ont influencées l'efficacité de la transmission. A plusieurs reprises, nous avons observé de fortes prévalences parasitaires chez les tiques lorsque les essais étaient réalisés au printemps, en comparaison de prévalences très faibles au cours de l'automne et de l'hiver. L'incubation des larves à 35°C préalablement au gorgement a permis d'optimiser le modèle et d'éviter les variations saisonnières. Les modalités de transmission mises en place, nous avons étudié le développement du parasite dans divers tissus de la tique. Nous avons montré la persistance de *B. microti* exclusivement au niveau des glandes salivaires chez les nymphes. La présence du parasite dans les glandes salivaires a été confirmée par la visualisation des sporozoites par FISH. De plus, nous avons observé l'infection de l'hôte par *B. microti* uniquement après gorgement complet des tiques infectées. Cela suggère que le développement des sporozoites matures – capables d'infecter un hôte naïf – dure 48 h après le début du gorgement. Nous avons donc mis en place et optimisé le modèle de transmission de *B. microti* de la larve à la nymphe. Cependant, la persistance du parasite uniquement dans les glandes salivaires des nymphes limite le rôle de l'immunité innée. Les molécules anti-microbiennes sont efficaces au cours de l'invasion du pathogène, particulièrement dans l'hémolymphe [120, 121, 123, 124, 284]. Ces résultats rendent le modèle établi inutilisable pour l'étude des interactions *Babesia*-tique avec l'utilisation de l'inactivation des gènes puisque cette technique n'est pas utilisable chez les larves.

Pour éviter cette complication, nous avons mis en place un modèle d'acquisition de *B. microti*. Ce modèle est basé sur l'infection de nymphes saines sur des souris de laboratoire infectées. L'acquisition du parasite par les tiques est un processus complexe qui comporte la reproduction sexuée dans le tube digestif du vecteur et l'invasion ensuite des glandes salivaires via l'hémolymphe [46, 51, 52]. En utilisant ce modèle, nous avons déterminé la chronologie de la dissémination de *B. microti* dans les glandes salivaires et tenté de visualiser les stades parasitaires dans les acini. Après plusieurs répétitions, nous avons confirmé la répétabilité et l'indépendance saisonnière des phases d'acquisition du parasite, contrairement au modèle précedent. Ce modèle est donc utilisé pour poursuivre les travaux sur les interactions mutuelles entre le parasite *Babesia* et son vecteur.

Les tiques possèdent des mécanismes anti-microbiens puissants permettant de réguler l'invasion par les pathogènes. Les organismes étrangers sont reconnus par les voies de signalisation immunitaire qui amplifient la réponse immunitaire par la production de molécules effectrices anti-microbiennes. Ce chapitre de la thèse décrit les trois voies de signalisation les mieux caractérisées orchestrant la réponse anti-microbienne, et évalue leur rôle dans l'acquisition des parasites *Babesia*. Nos travaux indiquent l'importance de deux NF-κB voies de signalisation immunitaire dans la régulation de l'acquisition parasitaire – la voie innée Toll et la voie Imd (déficience immunitaire). Les protéines TEPs (thioester containing protein) semblent aussi avoir un effet anti-*Babesia*. Les TEPs sont des homologues des facteurs du complément humain [120] et ont été préalablement identifiées dans la médiation de la réponse anti-microbienne d'*Ixodes ricinus* [120, 121, 124]. De plus, les TEPs ont été caractérisées comme des molécules effectrices en aval de la voie de signalisation Toll dans la réponse anti-malaria chez le moustique [277, 279]. Nous avons montré l'implication de deux TEPs dans la régulation de l'acquisition de *B. microti* par les tiques : la protéine TEP type complément et des protéines proches des macroglobulines (MCRs). Bien que nous n'ayons pas confirmé la connexion entre la NF-κB voies et les TEPs des tiques (expériences en cours), nous pouvons conclure que les tiques régulent activement l'invasion de *B. microti* via la réponse innée et par des mécanismes anti-microbiens impliquant le système du complément des tiques.

<u>Conclusion</u>

La thèse fournit une vue globale de mes recherches doctorales. Nous avons apporté un modèle optimisé et quantitatif du parasite parasite qui est utilisé ensuite dans plusieurs applications. Nous avons étudié le développement du parasite et particulièrement la transition vers la voie sexuée. Nous avons identifié le mécanisme de signalisation et d'immunité régulant l'invasion de *B. microti* chez la tique. Nous proposons une nouvelle voie dans la lutte anti-Babesia en validant le protéasome de *B. microti* et de *B. divergens* comme cible thérapeutique. Dans leur ensemble, les recherches présentées dans la thèse élargisse les connaissances actuelles sur la biologie du parasite *Babesia.*

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2012 – now **PhD student,** School of Doctoral Studies in Biological Sciences, Faculty of Science, University of South Bohemia, Ceske Budejovice, Czech Republic (dual Czech-French PhD. program)

2012 **RNDr.** (*rerum naturalium doctor*) in **Parasitology**

2009 – 2012 **MSc student,** Department of Parasitology, Faculty of Science, University of South Bohemia, Ceske Budejovice, Czech Republic
Thesis: Development of protective immune response in gastric mucosa of mice infected with *Cryptosporidium muris* and *Cryptosporidium andersoni*.

2006 – 2009 **BSc student**, Biology and Biomedical laboratory technique, Faculty of Science, University of South Bohemia, Ceske Budejovice, Czech Republic
Thesis: The immune response of mice to gastric cryptosporidium infections.

PROFESSIONAL EXPERIENCE

2012 – now Member of research team in **Laboratory of Vector Immunology** (head dr. Petr Kopacek) and member of research team in **Laboratory of Ticks Transmitted Diseases** (head dr. Ondrej Hajdusek), Institute of Parasitology, Biology Centre of CAS, Ceske Budejovice, Czech Republic

2011 – 2013 Member of research team of the project CZ.1.07/2.2.00/15.0361 (**Creation and Development Team Focused on Research and Teaching in the Field of Medical Biology**), Faculty of Science, University of South Bohemia, Ceske Budejovice, Czech Republic

2006 – 2012 Member of research team in **Laboratory of Veterinary and Medical Protistology** (head dr. Martin Kvac), Institute of Parasitology, Biology Centre of CAS, Ceske Budejovice, Czech Republic

PEER-REVIEWED PUBLICATIONS

Jirku Pomajbikova, K., M. Jirku, R. Sima, M. Jalovecka, B. Sak, K. Grigore, L. Wegener Parfrey (2017). "A benign helminth alters the host immune system and the gut microbiota in a rat model system. " PLoS One. Accepted.

Jalovecka, M., C. Bonsergent, O. Hajdusek, P. Kopacek and L. Malandrin (2016). "Stimulation and quantification of *Babesia divergens* gametocytogenesis." Parasit Vectors 9(1): 439.

Aase, A., O. Hajdusek, Ø. Øines, H. Quarsten, P. Wilhelmsson, T. K. Herstad, V. Kjelland, R. Sima, M. Jalovecka, P. E. Lindgren and I. S. Aaberge (2016). "Validate or falsify: Lessons learned from a microscopy method claimed to be useful for detecting *Borrelia* and *Babesia* organisms in human blood." Infect Dis (Lond) 48(6): 411-419.

Janotova, T., M. Jalovecka, M. Auerova, I. Svecova, P. Bruzlova, V. Maierova, Z. Kumzakova, S. Cunatova, Z. Vlckova, V. Caisova, P. Rozsypalova, K. Lukacova, N. Vacova, M. Wachtlova, J. Salat, J. Lieskovska, J. Kopecký and J. Ženka (2014). "The use of anchored agonists of phagocytic receptors for cancer immunotherapy: B16-F10 murine melanoma model." PLoS One 9(1): e85222.

Hajdusek, O., R. Sima, N. Ayllon, M. Jalovecka, J. Perner, J. de la Fuente and P. Kopacek (2013). "Interaction of the tick immune system with transmitted pathogens." Front Cell Infect Microbiol 3: 26.

Kvac, M., A. Kodadkova, B. Sak, D. Kvetonova, M. Jalovecka, M. Rost and J. Salat (2011). "Activated CD8+ T cells contribute to clearance of gastric *Cryptosporidium muris* infections." Parasite Immunol 33(4): 210-216.

Jalovecka, M., B. Sak, M. Kvac, D. Kvetonova, Z. Kucerova and J. Salat (2010). "Activation of protective cell-mediated immune response in gastric mucosa during *Cryptosporidium muris* infection and re-infection in immunocompetent mice." Parasitol Res 106(5): 1159-1166.

ATTENDED WORKSHOPS

2016 Workshop on Genomics, organized by evomics.org, Cesky Krumlov, Czech Republic

2015 EMBL introductory course: Fundamentals of Widefield and Confocal Microscopy and Imaging, Heidelberg, Germany

2015 Confocal Microscopy and Image Analysis Workshop, Ceske Budejovice, Czech Republic

CONFERENCES

2015 **oral presentation**, Tick and Tick Transmitted Diseases - workshop, Cesky Krumlov, Czech Republic

2014 **poster**, 1st biannual PARAFRAP conference, Les Embiez Islands, France

2014 **oral presentation**, 8th International Conference on Ticks and Tick-borne Pathogens (TTP8) and 12th Biennial Conference of the Society for Tropical Veterinary Medicine (STVM), Cape Town, South Africa

2013 **oral presentation**, Apicomplexa in Farm Animals, Kusadasi, Turkey

2013 **oral presentation**, Ticks Meet Mosquitoes – 1st traditional retreat workshop, Lipka, Czech Republic

2009 **oral presentation**, III. International *Giardia* and *Cryptosporidium* Conference, Orvieto, Italy

2009 – now **regular biannual oral presentation**, International Meeting of Czech Society for Protozoology, Czech Republic

HONORS, AWARDS AND FUNDING

2015 principal investigator of grant 103-002/2015/P provided by Faculty of Science, University of South Bohemia, Ceske Budejovice, Czech Republic; titled „Molecular interactions between the human parasite *Babesia microti* and tick vector"

2015 receiver of Boehringer Ingelheim Funds, Foundation for Basic Research in Medicine Travel Grant

2012 award of the rector (University of South Bohemia) for excellent achievement during the master study

2011 award for the best presentation in category of pre-gradual students, 43th International Meeting of Czech Society for Protozoology, Czech Republic

2009 – 2011 premium scholarship for outstanding study results, Faculty of Science, University of South Bohemia, Ceske Budejovice

CERTIFICATES AND SKILLS

2014 Certificate of competency according to § 17 of the Act No. 246/1992 coll. on Protection Animals against Cruelty in present statues at large. University of Veterinary and Pharmaceutical Sciences Brno and Central commission for Animal Welfare (CCAW).

2010 Certificate of Training on BD FACSCantoII^{TM} flow cytometer and BD FACSDiva 6.1 software

Languages: English (full professional proficiency, TOEFL ITP certificate), French (intermediate), German (intermediate), Czech (native).

PEDAGOGICAL ACTIVITY

2012 Biology of Science, University of South Bohemia, Ceske Budejovice, Czech Republic, lector for flow cytometry in project: EKOTECH – Multidisciplinary Education of Experts for the Use of Biotechnology in Enviromental Fields, CZ.1.07/2.3.00/09.0200

2010 – now Faculty of Science, University of South Bohemia, Ceske Budejovice, Czech Republic, lector of immunological methods in practise of Immunology course