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Piroplasmids and ticks: a long-lasting intimate relationship

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1. ABSTRACT

The tick-transmitted *Babesia* and *Theileria spp*. parasites are detrimental for animal health and cattle production in vast tropical and subtropical areas of the world. Additionally, human babesiosis increasingly raises public health concern. Most of the research on these piroplasmids has been focused on mammal-infecting stages, while the interaction with their tick vectors has been widely neglected. For millions of years, piroplasmids have been able to effectively exploit the tick milieu to carry out critical parts of their life cycle; including self propagation, sexual reproduction and recombination, development of infective forms capable of returning to their mammalian hosts through tick saliva, and in many cases, perpetuation into the next tick generation. Although piroplasmid colonization can seriously damage tick tissues and organs, innate immune mechanisms seem to be able to control these effects. This paper reviews the molecular interactions between ticks and piroplasmids from different perspectives. A deeper understanding of this interface might lead to the design of new control strategies.

2. INTRODUCTION

Apicomplexan parasites that are transmitted by ticks comprise *Babesia* and *Theileria* spp. Due to the existence of pear-shaped intraerythrocytic stages, they have been referred to as piroplasms (1,2). Traditional morphological and recent molecular investigations have substantiated that *Babesia* and *Theileria* spp. are close relatives and, correspondingly, they constitute the Order Piroplasmida (commonly: "piroplasmids") (3). In the following, the term *piroplasmid* will be used throughout to generically refer to *Babesia* and *Theileria* spp. parasites.

Piroplasmid infections are detrimental for animal health of a wide number of wild and domestic animals and are a major cause of economic losses in animal husbandry in tropical and subtropical areas worldwide (4). Furthermore, human babesiosis is being considered as an emerging zoonotic disease as a steadily increasing number of fatal infections of humans have been reported in recent years (5).

A unique morphological characteristic shared by piroplasmids with other apicomplexan protozoans is the presence of an apical complex: a rather intricate cellular apparatus consisting of conoid, rhoptries, micronemes (*Babesia*) or microspheres (*Theileria*), and other subcellular organelles (1,2). For *Babesia* spp. invasive stages, the apical complex serves as artillery to penetrate the host cell surface enabling the obligate intracellular lifestyle of these parasites. As a subgroup of the Phylum Alveolata, another prominent feature of apicomplexan protozoa is the existence of a peculiar cellular wrapping, the pellicle. Besides the outer cell membrane, the pellicle is composed of a primary subordinate layer of vesicles (alveoli) and a secondary layer of microtubules. The associated sublayer of microtubular structures (myosin/actin system) confers motility to the parasite and allows successful host cell invasion in the case of *Babesia* spp. (6) and other members of the Apicomplexa group. As both structures, the apical complex and the pellicle, are parasite-specific and thought to be vital for survival, their components are therefore at the forefront considered as targets for vaccines or antiparasitic drugs.

In respect to the above, *Theileria* shows significant variations to its taxonomic sister group (7). Most importantly, invasive parasite stages of *Theileria* spp. differ in the mechanism of host cell intrusion and correspondingly, in the function of the apical complex. Invasion into the host cell is mediated by a zippering mechanism between parasite and host cell membranes, in which apical complex organelles (rhoptries and microspheres) are not involved. The functional relevance of the latter seems to be restricted to the process of the intracellular establishment of the infection subsequent to invasion. For this reason, the search for potential vaccine candidates in the case of *Theileria* spp. has been focused predominantly on parasite surface proteins that may bind structures of the host cell membrane.

The invasion of mammalian erythrocytes by *Babesia* spp. has been dealt with in a number of studies (8,9). Thus, several parasite surface molecules that are apparently involved in erythrocyte invasion have been discovered (9,10). In addition, sialic acid present on erythrocyte surface proteins and cholesterol abundance in the erythrocyte membrane, as well as extracellular Ca^{2+} concentrations also play a critical role in parasite invasion (9,11,12). Furthermore, circumstantial evidence has been presented that *Babesia*-invasion pathways may be functionally redundant as has been demonstrated for *Plasmodium* (12,13,14). In contrast, studies of invasion of tick cells have been hardly ever addressed on a molecular level and are pending. A prerequisite to these studies would be the establishment of *in vitro* systems using tick cell lines to mimic the invasive phenomena that take place inside the ticks. This task can be envisioned as challenging, since even the straightforward propagation of the parasites in tick cell lines has not been successful so far (15). In the same way as the invasion of erythrocytes is considered a target for vaccine development, the invasion of tick cells may expose functional molecules that are vital for parasite survival and transmission.

3. TICK-PIROPLASMID INTERACTIONS FROM DIFFERENT PERSPECTIVES

3.1. A long voyage together

In this section, we will outline the current views on the phylogenetic history of Apicomplexa, ticks and vertebrates, which might provide insights into the co-evolution of interactions between piroplasmids and their invertebrate and vertebrate hosts at the molecular level.

Based on molecular comparison of 18S RNA genes, the basic phylogenetic relationships between *Babesia* and *Theileria* piroplasmids has been established by Allsopp *et al.* (3). Recently, further studies by Criado-Fornelio *et al.* (16) distinguished four clades of *Babesia* (archaeopiroplasmids, prototheilerids, babesids, and ungulibabesids) and one clade of *Theileria* parasites (theilerids). *Theileria* spp. are of monophyletic origin and are defined by their synapomorphic preerythrocytic propagation cycle in lymphocytes (schizogony) (2). In contrast, *Babesia* spp. constitute a paraphyletic group that can only be differentiated by molecular phylogeny, implying that characteristic molecular features of one *Babesia* parasite group may not always apply to another, and caution must be exercised to avoid inappropriate generalizations (16,17). By use of a molecular clock approach, the origin of the first piroplasmids (archaeopiroplasmids) has been dated to around 57 million years ago (mya). In this work, the molecular clock was calibrated by assuming concurrence of the appearance of the horse in Africa/Eurasia at about 7 mya with the tree node defining the babesid and ungulibabesid clades. The biological rationale for this calibration was based on the following assumptions: i) according to the inferred phylogenetic trees, the horse-infective *B. caballi* is the most ancient ungulibabesid, ii) in agreement with its worldwide distribution, diffusion of *B. caballi* between America, Eurasia, and Africa within its vertebrate host has only been possible during the Pliocene (7-2.5 mya). In accordance with the proposed molecular clock calibration, radiation of archaeopiroplasmids into the subgroups prototheilerids, theilerids and later into babesids, and ungulibabesids may have started around 25 mya (16).

Basal ixodid (hard tick) lineages have been exclusively identified in Australia suggesting they evolved about 120 mya after the isolation of this continent from Gondwanaland (18). As ticks (Ixodidae) are of monophyletic origin and since the oldest tick fossil of an argasid (*Carios jerseyi*) in New Jersey amber dates from 90 mya, this suggests that ticks had by then already diversified into main families (19,20). It has been proposed that the last common tick ancestor entertained a scavenger lifestyle feeding on body fluids of dead organisms like their phylogenetic sister group, the Holothyridae mites, before adopting a presumably more profitable blood-feeding lifestyle (21). This corresponds with recent evidence that anti-hemostatic mechanisms have been independently developed by different tick species during the above outlined time frame of tick evolution between 120 to 90 mya, possibly driven by the coinciding radiation of mammals and birds (22).

The origin of apicomplexan parasites is estimated to have occurred 820 to 350 mya, pre-dating by far the presumed origin of the invertebrate tick host as well as the radiation of mammals/birds (120 to 90 mya) (23). This raises the issue of which lineage represents the original monoxenous host of these parasites. As apicomplexan phylogeny correlates with the taxonomy of their invertebrate definitive host (e.g. piroplasmid/hard ticks, *Plasmodium*/mosquito), hard ticks most likely represent the primordial host of piroplasmid parasites (24). On this premise, the question arises as to when an ancient piroplasmid ancestor may have chosen a hard tick as its host. Given the scarce data and knowledge, an exact answer to this question remains elusive. However, on the premises that hard ticks originated apparently 120 to 90 mya and radiation of piroplasmids may have occurred 25 mya, it should have taken place within this time period.

In contrast, the vertebrate intermediate host seems to be integrated into a heteroxenous lifecycle in a more recent evolutionary time frame as also suggested by the observation that, within a given piroplasmid clade, a switch between different vertebrate hosts seems to have occurred relatively frequently (16,25). It may be assumed that the parasite is able to easily adapt to changes in the feeding behavior of the tick vector (16,26). Redundant erythrocyte invasion pathways that are implicated for *Babesia* sp. and have been demonstrated for *Plasmodium* sp. would be in accordance with this view as they suggest flexibility in the parasite's ability to infect different vertebrates (12,13,14). The above notion suggests furthermore, that as evolutionary adaptation between piroplasmid parasite and tick vector has likely spanned a much longer time, more intricate molecular interactions may be expected and realized between the latter than between piroplasmids and their vertebrate hosts. The molecular interface that may have been subjected to co-evolutionary forces between the invertebrate tick and the piroplasmid are outlined and discussed in the following section.

3.2. The journey through ticks

Successive stages of the life cycle of *Babesia sp*. and *Theileria sp*. parasites take place in different compartments of *Ixodidae* ticks. For this to occur, these parasites need to cross multiple barriers such as midgut and salivary gland epithelium, and, in *Babesia* parasites, due to their trans-ovarian mode of transmission, also the ovary epithelium. During the migration within tick tissues and cavities, the parasites undergo several metamorphic changes (1,2).

Following blood ingestion by the tick, intraerythrocytic parasites that reach the midgut are stimulated by unknown mechanisms to undergo gametogenesis, followed by gamete fertilization and the emergence of zygotes. Since these phenomena only take place in the tick gut, the presence of specific inducer molecules in the gut milieu can be expected. The closely related apicomplexan parasite *Plasmodium sp*. also undergoes sexual reproduction in the gut of its arthropod vector. When *Plasmodium*-infected blood reaches the mosquito gut, gametocytes, which are present in low numbers among the intraerythrocytic population of parasites in the mammalian host, transform into gametes that undergo sexual recombination and form zygotes, in a very similar way to what happens with *Babesia* and *Theileria spp*. These events are induced by a combination of three factors: (i) a drop of temperature with respect to that of the vertebrate host, (ii) a slight rise in pH and (iii) a substance present in the mosquito gut (27). The latter has been demonstrated to be xanthurenic acid, a heat-stable chromophore, which is a derivative of tryptophan metabolism (27,28). The mechanism by which xanthurenic acid induces gametogenesis is as yet not understood. Low concentrations of this compound can act together with pH to induce gametogenesis *in vitro* (27). Interestingly, xanthurenic acid has been found to stimulate the transformation of *B. bigemina* merozoites into sexual forms as well (29). In these experiments, incubation of intraerythrocytic parasites in an induction medium gave rise to the appearance of elongated stages with long projections that paired and fused, yielding diploid zygotes.

Segregation of satellite markers spanning the entire *T. parva* genome has been shown to be consistent with sexual recombination during tick-cattle passage (30). In an earlier study direct evidence for recombination has been demonstrated to occur between two distinct *T. parva* stocks following co-transmission through ticks (31). While the same is probably true for *Babesia sp*. parasites, its experimental demonstration is still pending (32). In addition, selection of piroplasmid subpopulations from heterogeneous field isolates is likely to happen during the tick life cycle stages.

Babesia and *Theileria* spp. parasites need to pass through the midgut epithelial cells for the perpetuation of their life cycle. The zygotes adhere to and invade midgut epithelial cells and transform into kinetes. This developmental step requires the expression of ligands on the surface of the zygote that interact with midgut cell specific receptors.

The identification of antigens containing neutralization-sensitive B-cell epitopes expressed on the surface of the sexual forms can lead to a control strategy that prevents transmission of the parasite within the vector. Again, research efforts carried out in *P. falciparum* parasites can be of benefit for *Babesia* and *Theileria* studies. It was observed that antibodies directed against Pfs230, a protein localized on the *P. falciparum* gamete surface, blocked transmission within mosquitoes (33). The search of the recently sequenced *B. bovis* genome (34) led to the finding of a Pfs230 homologous gene that is transcribed in sexual stages and intraerythrocytic forms of this parasite. Further characterization of this gene and the functional relevance of its product are currently under course (M. Florin-Christensen *et al*, unpublished observations).

Kinetes residing in gut epithelial cells are released into the hemocoel and then invade multiple tick tissues such as ovary epithelial cells and salivary glands.

The molecular interactions underlying these invasion processes are unknown. Some piroplasmids, such as *B. bovis*, invade the tick eggs, and develop to infectious stages within the salivary glands of the next generation of ticks, a process known as vertical transmission. Many other *Babesia* and all *Theileria spp*. lack vertical transmission and have to transmit the parasites to their respective mammalian host within the same tick generation. These different modes of transmission are likely due to a differential ability of the piroplasmid kinetes to invade tick eggs and might depend on the presence or absence of specific ligand/receptor interactions, which deserve future research efforts.

Following the invasion of the granular acini of salivary glands, the parasites undergo sporogony, and replicate to form sporozoite colonies. In the case of *T. equi*, replication has been demonstrated by quantification of parasites within the salivary glands of *Rhipicephalus microplus* and confirmed microscopically by the observation of *T. equi* colonies within the granular acini of salivary glands (35). Sporozoites express antigens on their cell surface required for erythrocyte invasion. An example of such a molecule is p67 of the sporozoites of *Theileria parva* that has been used in vaccination trials with significant success to prevent host infections (36,37).

3.3. Piroplasmid infections can be detrimental for tick health

Infection of ticks with *Babesia* or *Theileria sp.* parasites can have strong detrimental effects on these vectors. A study performed on *Boophilus decoloratus* ticks feeding on *B. bigemina*-infected splenectomized calves showed high mortality rates of engorged females and decreased viability in the eggs produced by surviving ticks as compared to ticks fed on non-infected calves, even though the blood parasitemia of the calves analyzed in this study was low (0.02-0.07%) (38). These results contrasted with previous studies performed on *Rhipicephalus* (*Boophilus*) *microplus* and *B. annulatus* that only showed enhanced mortality if blood parasitemia in *B. bigemina*-infected calves was higher than 20% (39). Thus, differences in vulnerability to infection by piroplasmids appear to exist between tick species.

In an early field study it was shown that at a given sampling site, the ratio of *T. annulata*-infected to uninfected ticks was usually lower when the ticks were heavily infected with the parasite (40). Based on this observation, it has been suggested that high levels of infection with this parasite may be detrimental for the invertebrate tick host. Likewise, *T. parva* seems also capable to cause considerable damage on *R. appendiculatus* ticks. In experiments carried out by Watt and Walker (41), ticks were applied on *T. parva*-infected and non-infected calves and detached engorged nymphs were collected daily, incubated for different times and examined. Clear pathological signs could be observed starting at day 9 after detachment. Moulting slowed down or stopped in the majority of infected ticks as compared to uninfected controls. Tick mortality dramatically increased coinciding with the rising of parasitemia in the calf, and reached 75-100% at peak parasitemia. A considerable number of the survivors detaching at this point were crippled, showing missing or distorted legs. Detailed examination of different tissues and organs of infected ticks revealed: (i) a vacuolated appearance in the basal area of the sessile digestive cells; (ii) a lack of structure in some areas of the gut epithelium accompanied by increased gut fragility; (iii) underdevelopment of salivary glands and deterioration of acini as evidenced by the appearance of pycnotic structures and large vacuoles. To measure reproductive performance, infected and uninfected ticks were applied to each ear of one rabbit. It was observed that infected females took longer to engorge and detach, the size of their bloodmeal was reduced, and they produced a smaller egg hatch. In addition, the hatched larvae numbers were significantly different between both groups (61% in uninfected compared to 12% in infected ticks).

The damages observed could be due to: (i) cell lysis caused by piroplasmids during invasion, (ii) degradation of vital tick macromolecules by parasite-released or membrane-bound enzymes, or (iii) a combination of both factors. Additionally, they could be caused by stress-related tick molecules that are released or produced as a response to piroplasmid invasion. However, as Watt and Walker comment, the reported effects of *T. parva* on *R. appendiculatus* ticks are unusual and appear to be an extreme example of the possible pathologies caused on ticks of this species by these parasites, since other infected ticks examined by the same authors showed no alterations or only slight abnormalities in the salivary glands. It can be concluded that variations in susceptibility among individuals within one tick species also exist. Supporting this view, it was observed that large individual differences in sporoblast numbers were observed upon experimental infection of *R. appendiculatus* ticks with *T. parva* under laboratory conditions (42).

3.4. How do ticks control piroplasmid infections?

In view of the possible deleterious effects that ticks may suffer due to piroplasmid invasion, it is natural to wonder how ticks control infections, keeping parasite numbers at levels that are compatible with their own survival and reproduction. And, likewise, how does the parasite ensure not to harm its invertebrate host irreversibly thus hampering its own transmission.

Current research is unraveling the existence of compounds that are part of innate immune mechanisms of ticks, protecting them from the uncontrolled invasion of bacteria, fungi or piroplasmids (43). It is thus possible to speculate that the observed differences in tick susceptibility towards infections by the latter could be connected to individual variations in the production of such defensive molecules.

Haemaphysalis longicornis is a hard tick found mainly in East Asia and Australia. It feeds on varied hosts and can transmit *Theileria spp*. and *Babesia ovata* to bovines, *B. gibsoni* to dogs and *Ricketssia japonica* to humans. This tick was recently found to produce a small protein of 5.8 kDa, that was named “longicin”, which has

similarities with a scorpion ion channel blocker and with several defensins from ixodid and argasid ticks (44). Expression of longicin can be detected throughout the life cycle of *H. longicornis*, including larval, nymphal and adult stages. The molecule is mainly expressed in the midgut and is significantly increased after a blood meal. Recombinant longicin was shown to display toxicity towards gram-positive and gram-negative bacteria, fungi and *in vitro* grown *T. equi* merozoites in a concentration-dependent fashion. Interestingly, the toxic activity of the molecule could be assigned to a 20 amino acid-long region of the longicin protein by experiments with synthetic peptides. Although the exact mode of action of longicin is not known, fluorescence confocal microscopy showed that the molecule attaches to the surface of *T. equi* merozoites, eventually leading to the lysis of the parasites. *In vivo* activity of this peptide on apicomplexan protozoa was tested in *B. microti*-infected mice. A significant reduction in parasitemia was observed upon administration of longicin. Moreover, pre-treatment of uninfected mice with longicin prior to challenge either abolished or significantly reduced infection with *B. microti*, depending on the administered dose. Importantly, no toxic effects (no erythrocyte lysis as obtained with other anti-babesial drugs) were observed in the treated mice, indicating a potential use of longicin in the development of new therapeutic approaches against *Babesia sp.* parasites (44,45).

Longicin and similar compounds that might be present in other ticks could thus participate in the regulation of the number of piroplasmids that remain and thrive in the tick organs and are finally transmitted to the vertebrate hosts. This hypothesis is further substantiated by the results of RNAi experiments. Longicin knock-down led to a significant reduction in the ability of *H. longicornis* adult ticks to feed and engorge when attached to *B. gibsoni*-infected dogs. These effects were accompanied by an increased number of *B. gibsoni* parasites in the midgut and the ovary, and as a consequence, the transmission of this parasite into the eggs was twofold increased (44).

Cystatin Hlcys2 is another molecule produced by *H. longicornis* ticks which is also able to arrest the growth of apicomplexan parasites (46). Cystatins are inhibitors of cysteine proteases, are widespread in plants and animals and have been implicated in the defense against pathogens (47,48,49). Maximal transcription of the Hlcys2 gene was found in the midgut and hemocytes of these ticks, and was low in salivary glands, fat body and ovaries. Transcription occurred in all stages but was maximal in adults. Most noteworthy, transcription increased almost 17 times four days after adult ticks started feeding on rabbits. In addition, ticks fed on a *Babesia gibsoni*-infected dog showed a 1.8 fold increase in Hlcys2 transcription as compared to ticks fed on an uninfected dog. The possible toxic activity of Hlcys2 on piroplasmids was tested on *in vitro* cultured *B. bovis* merozoites. Parasites grown in the presence of Hlcys2 were arrested in their growth in a concentration-dependent fashion, although total growth inhibition was not observed (46).

Cystatins have also been described in other ticks such as *R. microplus* (50), *Ixodes scapularis* (51) and *Amblyomma americanum* (52) but their biological roles and possible effects as defensins have not yet been explored. Cystatins may fulfill different physiological roles in these ticks, such as facilitating blood feeding and regulation of their own cysteine proteases. In addition, they may be involved in the inhibition of piroplasmid cysteine proteases, thus limiting parasite multiplication in their organs and avoiding degradation of critical proteinaceous reservoirs, such as vitellin, the main nutrient of tick eggs, upon piroplasmid colonization.

Genes encoding cysteine proteases have been described for *T. annulata* (53), *T. parva* (54, 55), *T. sergenti* (56), *T. equi* (57), *B. caballi* (57), *T. orientalis* (58) and *B bovis* (10). The roles of piroplasmid cysteine proteases are still unknown although they have been postulated to participate in pathogenicity, erythrocyte invasion and degradation of hemoglobin. Secondary analysis of *B. bovis* putative cysteine protease predicted a membrane-bound protein with an exposed catalytic site (Mesplet, M. *et al*, manuscript in preparation), which would allow the degradation of proteins present in the parasite extracellular medium. Cysteine protease inhibitors were shown to hinder *in vitro* growth of merozoites of *T. equi* (57) and *B. bovis* (59), suggesting that these enzymes are basic for the survival of these parasites in their intraerythrocytic stage. These types of studies have been centered so far in the parasite-mammalian host interface. The importance of cysteine proteases for the tick-associated piroplasmid stages and their participation in invasion of tick cells, as well as their pathogenicity towards tick tissues are yet to be unraveled.

Innate immunity (43) has been recognized as a major defense mechanism of arthropods against microbial infections. Since the discovery of innate immune receptors such as toll-like receptors and other pattern recognition receptors (PRRs) that bind foreign microbe-specific molecular structures (e.g. pathogen associated molecular patterns, PAMPs), the importance of innate immune mechanisms of plants, arthropods (*Drosophila* sp.) and other invertebrate organisms is being intensively investigated. However, research on the effect of the innate immune mechanisms of ticks to control parasites they transmit has been relatively scarce. The availability of genome information of some pathogen-transmitting tick species might facilitate the isolation and functional characterization of PRRs, enabling the in-depth characterization of tick defense mechanisms against piroplasmid infections.

In addition, transcriptomic and proteomic approaches can lead to an increased understanding of the molecular cross-talk between ticks and piroplasmids. These approaches have been recently applied to search for changes in protein expression in ovaries of *R. microplus* ticks infected with *B. bovis* (60) and transcription of genes in the salivary glands of female *R. appendiculatus* ticks upon invasion with *Theileria parva* (61).

In the first case, a significant increase in the amounts of a number of tick small proteins was observed in tick ovaries upon infection with *B. bovis* (60). Although further functional investigation of these proteins is still needed, some of them shared characteristics with invertebrate antimicrobial peptides. Together with the above mentioned longicin from *H. longicornis*, these results are suggestive of a common strategy among ticks that involves increased synthesis of defensins in response to piroplasmid invasion. A Kunitz-type serine protease inhibitor was also observed to be up-regulated in *R. microplus* ovaries upon *B. bovis* invasion. This result is particularly interesting given our recent finding of serine protease encoding genes in the genome of this parasite (10, Mesplet, M. *et al*, manuscript in preparation). Serine proteases have been implicated in the invasion of erythrocyte by *B. divergens* parasites, after *in vitro* growth experiments in the presence of specific inhibitors (62). Although no studies are available on the possible action of this type of enzymes in the invasion of tick cells, it may be hypothesized that a serine-protease inhibitor constitutes a mechanism to control the action of parasite enzymes. Lysozyme, on the other hand, was down-regulated in infected ovaries. This enzyme is known to have antibacterial and digestive functions in different organisms, and was shown to be up-regulated in the soft tick *Ornithodorus moubata* after a blood meal (63). It is not yet understood if and how lysozyme can interact with *B. bovis*, although it has been suggested that it prevents invasion of tick tissues by these parasites (64). The observed down-regulation of lysozyme expression upon ovary infection by *B. bovis* might be indicative of an adaptive mechanism of these parasites to invade tick ovaries and eggs and thus achieve transmission to the next tick generation.

Although major changes in the transcription of *R. appendiculatus* abundantly transcribed genes could not be detected upon invasion of salivary glands with *T. parva*, some genes encoding glycine-rich proteins appeared to be up-regulated, but further studies are needed to ascertain their exact functional role and the significance of this observation (61).

3.5. Tick saliva-camouflage of sporozoite invasion

The capability of ticks to blood feed on vertebrates demanded sophisticated adaptations to ensure, on one hand, continuous blood flow and, on the other, evasion of the immune defense mechanisms of the host. Although these adaptations are also present in other arthropod-pathogen vector systems (e.g. sandfly/*Leishmania* spp, mosquito/*Plasmodium* spp.), ticks are long feeders that are attached to the host for 3 to 10 days which calls for the necessity of additional adaptations, such as: i) the need to stay undetected as the long attachment period renders them particularly sensitive to grooming of the vertebrate host; and ii) the need to cope with immediate innate and late specific adaptive immune defense mechanisms, as well as wound healing reactions of the host. These necessities have led to the development of a plethora of highly efficient substances that ultimately assist them in their feeding success (51). In a recent investigation of the sialome of the salivary glands of the tick *Ixodes scapularis,* 470 putative secreted proteins were discovered that could be grouped into more than 25 different protein families (65). The function of most of these proteins and/or protein families is yet unknown and awaits investigation. Considering that only 70 putative secreted proteins from the salivary glands of the *Plasmodium*-transmitting mosquito *Anopheles gambiae* have been found so far (66), it appears that the complexity of saliva is considerably higher in ticks than in mosquitoes. Furthermore, a substantial larger number of different vasodilatory and immunomodulatory activities has been reported for hard ticks than for any other arthropod vectors (67). Evidence has been presented that the main evolutionary mechanism that resulted in this large number of different proteins has been gene duplication and subsequent positive selection leading to diversification into new functions (21,68).

Specifically, anti-hemostatic proteins that are secreted by the tick exert their function by inhibiting platelet activation and aggregation, by inhibition of factors of the blood coagulation cascade, and by fibrin (ogen)olytic proteins. As ticks are pool feeders, these factors ensure blood fluidity in the hematoma from where the blood is taken up, during feeding and after its ingestion into the tick gut. In addition, potent vasodilatory substances ensure a continuous inflow into the hematoma and its interconnection with the blood circuit. The active intermittent secretion of the tick saliva containing these factors into the hematoma ensures a continuous blood flow and, likewise, an effective transmission of the pathogen into the host (69).

Another group of factors secreted by the tick have been shown to interfere with a multitude of immune effector mechanisms exerted by the host, by inhibiting inflammation, complement activation, antibody response, cytokine modulation, and proliferation of T lymphocytes. Other immune cells that are inhibited in their function comprise neutrophils, macrophages, NK cells, dendritic cells and B-cells. Inactivation of anaphylatoxins and inhibition or binding of histamine/serotonin may prevent pain and thus help to keep the blood-feeding tick undetected by the host (67,70).

The above portrayal plainly demonstrates that, at this stage of the life cycle, pathogen and blood-feeding tick have mutual interests since immunosuppressive agents, which allow the tick to feed may facilitate and ensure the establishment of pathogen infection. Accordingly, it has been shown for a number of arthropods that their saliva facilitates pathogen transmission and establishment of infection (70,71,72). Molecular mechanisms that facilitate and ensure transmission of the prokaryotic pathogen *Borrelia burgdorferi* by *Ixodes scapularis* to their vertebrate host have been reported (73,74). In parallel, it has been shown that the infectivity of *T. parva* sporozoites was enhanced between 30 to 60 % after preincubation of bovine lymphocyte target cells with tick salivary gland extract or with proinflammatory cytokine IL-2 (75). Similar mechanisms have not been identified yet for *Babesia spp.* or other *Theileria* spp. parasites. This might be due to the lack of investigations, but, in addition, the high complexity

of these eukaryotic parasites should endow them with additional possibilities to manipulate their hosts. It might thus even be conceivable that the pathogen assists the tick in the acquisition of its blood meal, either by manipulating its behavior as described for *Plasmodium* (see next chapter) or by the secretion of molecules that have synergistic functions with the ones described above for the tick vector.

3.6. Can piroplasmids manipulate their tick vectors?

The possible occurrence of parasite manipulation of its host is one of the many aspects of tick-piroplasmid interactions that remain unexplored. This intriguing phenomenon happens when a parasite enhances its own transmission by altering the behavior of its host or vector, through direct or indirect means. The former takes place when the parasite secretes or excretes a neuroactive substance that results in changes of host behavior. While in the latter, the presence of a parasite influences the host development, intermediate metabolism and/or immunity, leading secondarily to an alteration of host behavior (76). A rich variety of manipulation mechanisms have been generated by different parasites of invertebrates. The example that is closest to tick-piroplasmid relationships is provided by *P. falciparum* in its interaction with its mosquito vector. On one hand, *Plasmodium*-infected mosquitoes were shown to feed on their vertebrate hosts more frequently than uninfected mosquitoes. Sporozoites present in the salivary glands hamper efficient feeding by inhibition of the mosquito apyrase and, as a consequence, mosquitoes need to probe more times in order to obtain a given amount of blood (77). On the other hand, it was observed that human subjects infected with malaria attract mosquitoes twice more efficiently than uninfected individuals (78). The mechanism underlying this manipulation is unknown, but it is likely that the parasites change the infected individual's breath or body odor. Taken together, both phenomena show an incredible exploitation of the vector behavior by the *Plasmodium* parasite that leads to its increased transmission. Although unexplored so far, the existence of manipulation phenomena in tick-transmitted apicomplexan protozoa is conceivable, and its study would provide a fascinating viewpoint of the tick-piroplasmid interactions.

4. CONCLUDING REMARKS

We have analyzed the possible molecular interactions between ticks and the piroplasmids they transmit from different perspectives, although the questions by far surmount the answers. This largely underdeveloped research field is bound to expand with the increasing availability of tick and parasite genome information. In addition, the efforts applied to study *Plasmodium*-mosquito molecular interactions might have a catalytic effect on the research on *Babesia* and *Theileria* relationships with their tick vectors. Ticks and tick-borne pathogens are a permanent threat to animal and human health, and the current global warming scenario ensures the permanence and probable intensification of this challenge in the coming years. Rationally-designed vaccines and therapeutic agents that could simultaneously limit ticks and transmission of piroplasmids would be a highly desirable control option, in view of the drawbacks and limitations of the currently available management methods for ticks and tick-borne parasites. This kind of concerted approach will only be feasible after a much better understanding of the molecules and events that rule the tick-piroplasmid interface.

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