Trends in Parasitology

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Review

Babesia Life Cycle – When Phylogeny Meets Biology

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Although *Babesia* represents an important worldwide veterinary threat and an emerging risk to humans, this parasite has been poorly studied as compared to *Plasmodium*, its malaria-causing relative. In fact, *Babesia* employs highly specific survival strategies during its intraerythrocytic development and its intricate journey through the tick vector. This review introduces a substantially extended molecular phylogeny of the order Piroplasmida, challenging previous taxonomic classifications. The intriguing developmental proficiencies of *Babesia* are highlighted and compared with those of other haemoparasitic Apicomplexa. Molecular mechanisms associated with distinctive events in the *Babesia* life cycle are emphasized as potential targets for the development of *Babesia*-specific treatments.

Highlights

Evolutionary diversification of vertebrate hosts is the major driving force of Piroplasmida phylogeny.

Remarkable diversity of the genus *Babesia* represents one of the greatest challenges for the development of generic antibabesial therapeutics.

Specific events of the *Babesia* life cycle represent mandatory adaptations of *Babesia* to the tick vector.

Analogies between *Plasmodium* and intra-tick development of *Babesia* opens avenues for developing novel intervention strategies.

Babesia: A Parasite of Veterinary and Medical Relevance

Babesia is an apicomplexan tick-transmitted haemoparasite that has developed unique strategies in order to complete its life cycle and transmission. Essential reproductive goals are: (i) to perpetuate its parasitic existence by propagation, and (ii) to guarantee host-to-host transmission through specialized infective stages. Both objectives are mediated by the combination of two asexual reproduction cycles and one sexual reproduction cycle, which alternate between the vertebrate host and the tick vector. The *Babesia* life cycle follows the archetypal developmental scheme of members of the Apicomplexa, including three successive phases: **merogony**, **gamogony**, and **sporogony** (see Glossary and Figure 1) [1,2]. However, *Babesia* parasites have evolved novel strategies resulting from the need to adapt to the feeding and molting of their tick vectors, the definitive hosts [3]. Because hard ticks (Ixodidae) feed only once per instar [4], *Babesia* has developed the ability to persist through successive tick developmental stages, referred to as **transstadial transmission** [5]. An additional strategy of *Babesia* perpetuation is **transovarial transmission**, allowing for the spread of the parasite from a single maternal tick to thousands of offspring [5]. Transovarial transmission is considered to be an unusual mode of propagation within the phylum Apicomplexa. Within the order **Piroplasmida**, it is exclusively displayed by the evolutionary lineage of ***Babesia* sensu stricto** (Figure 2). The *Babesia* capacity for long-term occupation of ticks via transovarial transmission translates into parasite reservoirs during the absence of the vertebrate host [6]. The proficiency of transmission, together with the worldwide distribution of tick vectors, makes *Babesia* the most common parasite of free-living and/or domestic animals [7]. Bovine babesiosis has a high economic impact on the livestock industry, with 1.2 billion cattle at risk of infection [8]. Humans are accidental hosts of *Babesia* [6], but many aspects – for example, increasing travel activity, blood transfusions, etc. – contribute to human babesiosis as a serious public health concern [9,10]. Human babesiosis is treated using a combination of antibiotics and antimalarial drugs [9]. A very effective treatment for mild to moderate cases can be achieved by the use of atovaquone and azithromycin [9]. However, in immunosuppressed patients the necessary prolonged drug

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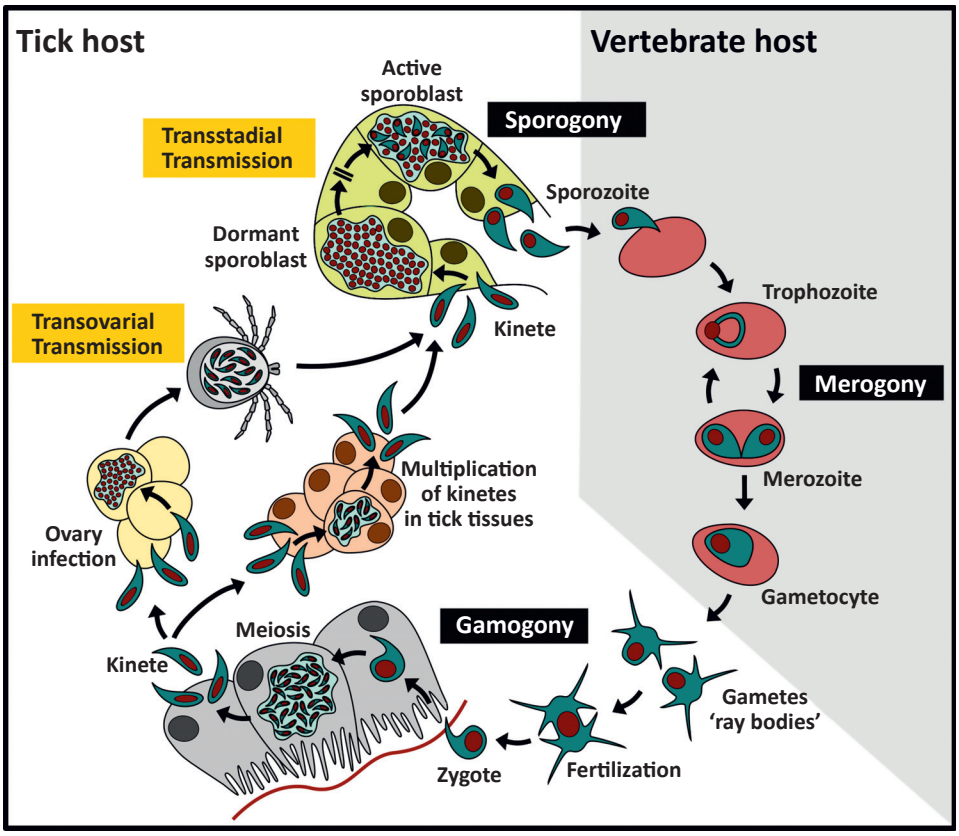

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Figure 1. Life Cycle of *Babesia* sensu stricto. The vertebrate host is infected by the infective sporozoite during tick feeding. Sporozoites invade red blood cells (RBCs) of the host where they develop into trophozoites. Trophozoites divide into merozoites via the process of merogony (binary fission), subsequently egress, and re-infect other host erythrocytes. Gamogony (sexual reproduction) is initiated by the sexual commitment of merozoites to intraerythrocytic gametocytes, the transient stage that infects the tick vector (definitive host). Gametocytes that are taken up into the tick gut lumen during feeding further develop into gametes. *Babesia* gametes, referred to as ray bodies or Strahlenkörper, fuse and create a motile zygote termed the ookinete. The ookinete penetrates the peritrophic matrix (semipermeable noncellular membrane surrounding the tick midgut lumen) and invades the tick gut cells. Once inside the epithelium, the zygote undergoes meiotic division, resulting in the production of kinetes. Kinetes disseminate via the tick body fluid (haemolymph) to peripheral tick tissues, including ovarian cells. The invasion of tick ovaries results in the infection of eggs and leads to *Babesia* transovarial transmission. In parallel, primary kinetes invade other tick organs and, via schizogony (multiple fission), multiply to create secondary kinetes that invade tick salivary glands. Inside salivary gland cells, invading kinetes develop into a multinucleated syncytium (sporoblast), which stays dormant during the tick ecdysis, ensuring transstadial transmission of the parasite. Once the next infected tick instar initiates blood-feeding on a naive host, the sporoblast is activated and multiple infective sporozoites are continuously produced by the process of sporogony and released into the host bloodstream.

application often results in the generation of resistance mutations, leading to relapse of disease and chronic infection [11]. An alternative therapy, using a combination of clindamycin and chinin, has been recommended against severe babesiosis [10] but the treatment is often accompanied by toxic effects [12]. In addition, the effectiveness of this treatment has never been tested in clinical trials. Hence, the development of novel and well tolerated drugs based on *Babesia*-specific molecular targets is highly desirable in the face of an increasing number of immunocompromised patients that need prolonged treatment [10,11].

Glossary

ApiAP2: a gene family of transcription factors regulating sexual commitment.
Apical complex: a multiorganelle apparatus of cytoskeletal structures and associated organelles mediating host cell invasion by parasites of the phylum Apicomplexa.
***Babesia* sensu lato:** *Babesia* species of different evolutionary lineages lacking the ability of transovarial transmission.
***Babesia* sensu stricto:** one of the evolutionary lineages of piroplasmids displaying transovarial transmission, also referred to as the 'true' *Babesia* species.
C1A cysteine-proteases: papain-like proteolytic enzymes (aka proteinases, proteases, peptidases) regulating physiological functions – invasion, egress, and nutrition.
CCp genes: genes encoding adhesive proteins of the LCCL (*Limulus* coagulation factor C) family. These proteins are associated with parasite sexual commitment and gamogony.
Gamogony: sexual reproduction that allows for the recombination of genetic material between gametes.
Kinases: enzymes that add phosphate groups to other molecules (proteins, lipids, or nucleic acids) – such signals control critical cellular processes such as cell signaling, transport, and division.
Merogony: asexual multiplication (binary fission) of merozoites within vertebrate RBCs.
Peritrophic matrix: a peptidoglycan-based semipermeable noncellular membrane surrounding the inner surface of the tick midgut lumen.
Piroplasmida: a group of piroplasmids consisting of three genera *Babesia*, *Theileria*, and *Cytauxzoon*.
Schizogony: asexual reproduction characterized by multiple nuclei divisions, forming a multinucleated syncytium followed by budding and cytokinesis.
Sexual commitment: parasite transition from asexual stages (merozoites) to sexual stages (gametocytes) in the bloodstream of a vertebrate host.
Sporogony: schizogony-analogous propagation inside the cells of tick

salivary glands resulting in the production of infective sporozoites.
Transovarial transmission: the ability of 'true' *Babesia* (sensu stricto lineage) to rapidly disseminate thousands of offspring from a single infected tick female through their reproductive organs and embryos into the next tick generation.
Transstadial transmission: the transmission of the parasite between two or more tick instars (larva via nymph via adult).

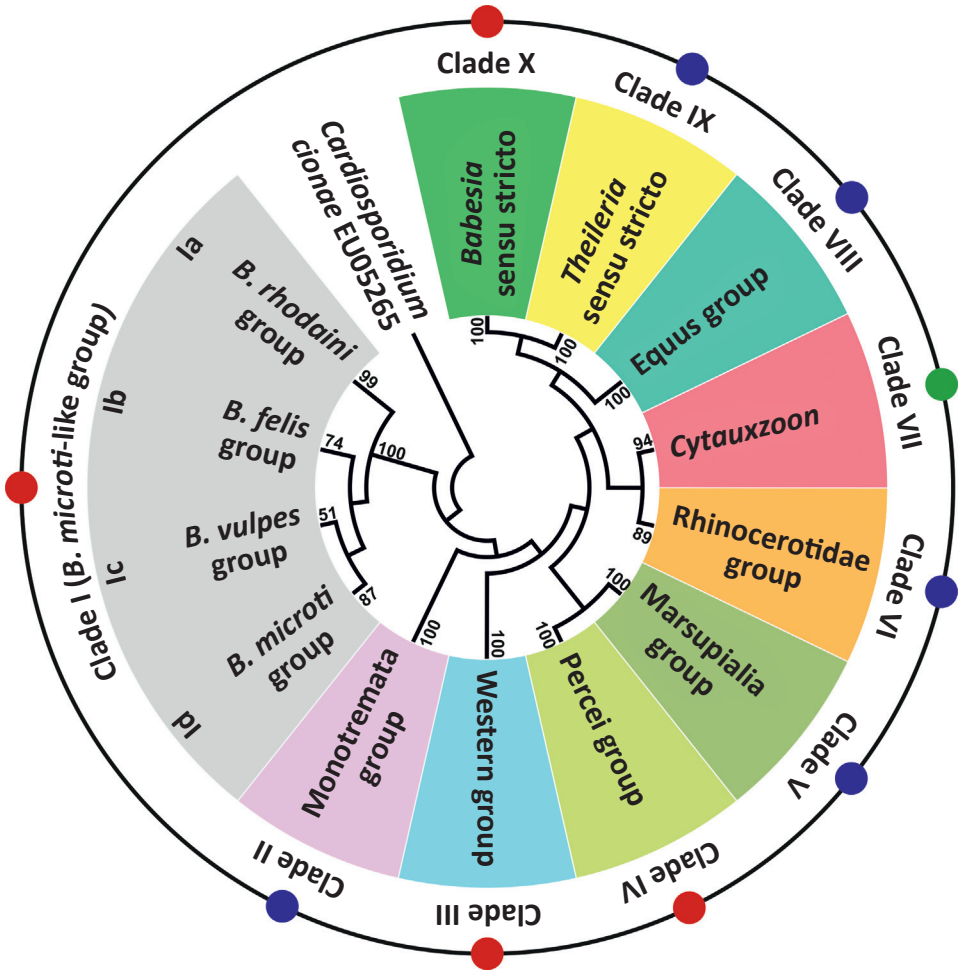

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Figure 2. Molecular Phylogeny of Piroplasms. A neighbour-joining phylogenetic tree of 18S rRNA gene sequences of *Babesia*, *Theileria*, and *Cytauxzoon* species is shown. Piroplasmid clades are well defined, as demonstrated by high bootstrap values. Piroplasmid lineages (for a detailed description see Table 1) of clade Ia (Rodentia, Macaca), Ib (carnivores: Canidae, Mustelidae), Ic (Rodentia), Id (Felidae), II (Monotremata), IV (Marsupialia), V (Aves), VI (Rhinocerotidae), VII (carnivores: Felidae, Ursidae, Herspestidae) VIII (Equidae), and IX (Bovidae, Cervidae) specifically infect indicated mammalian families/orders and/or birds. In contrast, piroplasmids of clade III (Bovidae, Cervidae, Felidae, Herpestidae, Canidae) and, in particular, of clade X (Marsupialia, Rodentia, Canidae, Mustelidae, Giraffidae, Bovidae, Cervidae, Aves) infect a large variety of mammalian families/orders and/or birds, suggesting that *Babesia* sensu lato of the Western group and *Babesia* sensu stricto have switched frequently between mammalian families, orders, and bird hosts. Red dots = clades comprising piroplasmids referred to as *Babesia*; blue dots = clades comprising piroplasmids referred to as *Theileria*; green dot = the clade comprising *Cytauxzoon* species. The alignment was comprised of 184 sequences and had a total length of 1638 bp. Bootstrap values based on 1000 replicates are displayed next to the branches. The tree has been rooted using the sequence of *Cardiosporidium cionae*.

Merogony: An Intraerythrocytic Style of Life

Merogony in *Babesia* results in the propagation of pyriform merozoites within host red blood cells (RBCs) and may lead to long-lasting persistence in natural vertebrate hosts and humans [6]. Sporozoites are injected into the host bloodstream during the feeding of an infected tick individual, and sporozoite invasion of RBCs initiates merogony (Figure 1). Intracellular sporozoites develop into trophozoites, which further divide into merozoites able to egress and

Box 1. Apical Complex: Cellular Apparatus Mediating Host Cell Invasion

The capacity for cellular invasion by the Apicomplexa is mediated by a unique multiorganelle apparatus – the apical complex – representing a key structure for the evolutionary success of apicomplexans. The apical complex is located at the anterior cellular pole of all invasive stages such as sporozoites and merozoites, and in the case of *Babesia* also of intra-tick kinetes (see Figure 1 in main text). Specialized secretory organelles govern parasite orientation and penetration to the host cells via secretion and specific cleavage of proteins, and the utilization of a myosin–actin motor complex in the invasion process [82,83]. The apical complex is believed to have evolved from the flagellum of free-living photosynthetic algae ancestors of the Apicomplexa [84]. Although its core structure is highly conserved, piroplasmids exhibit a reduced apical complex composed of a species-dependent number of rhoptries, a reduced number of micronemes, and only one polar ring; furthermore, the conoid is absent (Aconoida) [1,13]. *Babesia microti,* a representative of *Babesia* sensu lato (see Figures 2 and 3 in main text), possesses the most reduced apical complex, comprising a single large rhoptry, while polar rings and subpellicular microtubules are absent [64]. Intriguingly, a substantial apical complex reduction (e.g., the lack of polar rings) can be seen in *B. microti* and colpodellids – the free living sister group of parasitic Apicomplexa. These single-cell predators use the apical complex for feeding on algae or protozoa by myzocytosis [85], and, due to their considerable evolutionary distance, we can assume that the apical complex reduction of both, *B. microti* and colpodellids, represents a case of convergent evolution. Nevertheless, a case of human infection with *Colpodella* spp., resembling human babesiosis caused by *B. microti*, has been recently reported [86]. All species of *Babesia* employ a single large protein-secreting organelle known as the 'spherical body', which appears to be analogous to the dense granules of other Apicomplexa, and its role is to coordinate the formation of the parasitophorous vacuole, a compartment surrounding the intracellular parasite [13,87]. However, in contrast to many other members of the Apicomplexa (e.g., *Plasmodium, Toxoplasma*), in which the parasitophorous vacuole persists at least until the intracellular parasite multiplication is completed, the *Babesia* parasitophorous vacuole disintegrates shortly after host cell invasion [87], and *Babesia* parasites thus reside directly within the cytoplasm of the infected host cells [13,15,88].

reinfect naïve RBCs. The process of RBC invasion is mediated by proteins secreted by the **apical complex**. Interestingly, in the case of *Babesia*, this archetypal cellular apparatus of apicomplexans displays a remarkable reduction (Box 1) [3,13], suggesting alternative molecular mechanisms of invasion and establishment inside the host cell. In particular, the proteins secreted from the spherical body – an organelle analogous to the dense granules of other apicomplexan parasites – appear to be remarkably altered as no significant homology to other apicomplexan proteins has been identified for *Babesia bovis* spherical body protein 4 [14]. In contrast, the structural analyses of *Babesia* proteins associated with rhoptries and micronemes suggest a conserved function across *Babesia* species, and homologues occur in *Plasmodium* (summarized in [15]).

Free and internalized merozoites display a mosaic of integral membrane proteins on their surface, collectively referred to as merozoite surface antigens (summarized in [15]), believed to play a predominant role in parasite propagation [16,17]. The family of variable merozoite surface antigens (VMSAs) belongs to the most studied surface proteins and appears to be involved in the invasion process [18]. In addition, the intraerythrocytic stages of *Babesia* also cause antigenic variation of the erythrocyte's surface. The parasite has been shown to evade host immunity through consecutive expression of a large family of parasite-derived variant erythrocyte surface antigens (VESAs) [19]. Some of these variant surface and secretory antigens have been proposed as vaccine targets or diagnostic tools [17,20]. The proposed evolutionarily conserved regulatory protein domains of variant antigens might constitute common targets for all Piroplasmida species [21]. In our view, an increased effort to study merozoite-expressed variant proteins would allow a more profound understanding of host cell surface alterations by *Babesia* parasites and reveal possibilities for specific clinical applications against babesiosis.

Sexual Commitment: From Host to Vector

Babesia **sexual commitment,** or outset of gamogony by formation of the gametocytes, starts in the bloodstream of the vertebrate host (Figure 1) and is completed upon ingestion of infected RBCs by the tick vector [3,5]. A developmental decision assures generation of nondividing

sexual stages, which are morphologically indistinguishable from simultaneously occurring asexual blood stages [22]. This process contrasts with the synchronized intraerythrocytic development of *Plasmodium* [23] and likely corresponds to the several days long tick feeding providing a continuous ingestion of RBCs containing gametocytes. *Babesia* sexual commitment is governed by as yet unknown mechanisms. In *Plasmodium*, the sexual differentiation is regulated by the combination of genetic and environmental factors [24], and also for *Babesia divergens* the application of stressors – high host parasitaemia or drug treatment – resulted in increased production of gametocytes [22]. Recently, transcription factors encoded by the AP2 gene family (**ApiAP2**) have been shown to regulate the sexual development of *Theileria*, a sister genus of *Babesia* [25]. Since these proteins are conserved in *Babesia* and *Theileria* species [21], we assume that they have functionally similar roles in the Piroplasmida. One of the well-described antigenic markers of gametocytes are proteins encoded by the **CCp genes**, which are highly conserved among the Piroplasmida [26,27] and other apicomplexans [28]. Since some of the CCp proteins play an essential role in the transmission of *Plasmodium* to the mosquito vector [28,29], we would like to highlight their putative potential as *Babesia* transmission-blocking vaccine antigens.

Gamogony: Sexual Development in the Tick Gut

Once *Babesia* gametocytes appear in the gut of a feeding tick, they begin their metamorphosis into gametes by the process of **schizogony**, a multiple fission [30–32]. Due to their specific morphology, babesial gametes are referred to as Strahlenkörper or spiky-rayed bodies [5] (Figure 1), that are remarkably different to the archetypal micro- and macrogametes of other apicomplexans [1], including *Babesia*-related *Theileria* or *Hepatozoon* [5,33]. Gamogony is mediated via syngamy (fusion of gametes) that leads to the formation of a motile zygote – ookinete – subsequently penetrating the **peritrophic matrix** to invade tick gut cells [3]. Zygotes of most apicomplexans form a surrounding protective wall resulting in the oocyst, which – after meiotic division – is filled with gradually maturing sporozoites [1,34]. In contrast, the zygote of *Babesia* does not form an oocyst. Instead, it undergoes meiotic division inside tick gut cells resulting in the production of unicellular kinetes. Kinetes disseminate through the tick haemolymph to peripheral tick tissues where they undergo further schizogony-analogous propagation prior the production of sporozoites (sporogony, Figure 1) [3].

Sporogony: Generation of Multiple Infective Sporozoites

Sporogony in *Babesia* occurs in the tick salivary glands (Figure 1). To ensure parasite survival during the constructional changes of tick molting, invading kinetes arrest in the form of a polymorphous syncytium – the dormant sporoblast. Once the infected successive tick instar attaches to another vertebrate host, the dormant sporoblast becomes activated [3,32,35] and infective sporozoites asynchronously bud off from the syncytium and are continually released into the host blood during tick feeding [32,35]. Major differences between *Babesia* and *Plasmodium* sporogony originate from the specific adaptation of each parasite to the contrasting feeding strategies of their respective vectors – hard ticks feed for days and once per instar, while mosquitoes feed rapidly within minutes and feeding can be repeated within the same instar. In contrast to *Babesia*, plasmodial sporogony occurs inside the oocyst within the cells of the basal lamina of the mosquito gut [36]. Fully matured sporozoites migrate from the oocyst through the mosquito haemolymph and are collected in salivary gland ducts of the mosquito, ready for immediate transmission during rapid feeding [36]. Besides this difference, babesial and plasmodial sporozoites share analogous features such as the ability to infect vertebrate cells during vector feeding and the high conservation of key regulatory domains of DNA-binding proteins [21]. Importantly, the transmission of sporozoites to the vertebrate host is a bottleneck in the life cycle of the parasite. Thus, molecules mediating sporogony and sporozoite transmission may represent

suitable therapeutic targets. This is well documented by the use of vaccines based on the surface antigens of plasmodial sporozoites [37] that are able to effectively reduce relapses of malaria. We propose that an analogous approach may be effective against babesiosis. Moreover, we would like to postulate that the efficacy of molecular vaccines might be further enhanced when vaccine formulations are based on a combination of several stage-specific antigens, including sporozoite, merozoite, and potentially also gametocyte-surface antigens. Recent sequencing and proteomic approaches in *Theileria* [38,39] are a great example of initial steps leading to the identification of stage-specific antigens of piroplasmids.

Transstadial Transmission: A Prerequisite for Transmission by Ticks

Dormant *Babesia* sporoblasts enable transstadial transmission [3,5,32], a common phenomenon among all tick-transmitted pathogens [4]. The driving force for such an adaptation is the fact that tick-transmitted pathogens need to persist through tick molting in order to infect naive hosts during the on-host feeding of the next tick instar. Besides piroplasmids [3,5], *Hepatozoon* (haemogregarines) represents another species of tick-borne apicomplexans capable of transstadial transmission [33,40]. In contrast to the intracellular sporoblast of *Babesia*, *Hepatozoon* survives tick ecdysis in the extracellular oocyst filled with sporoblast [33]. The wall of the oocyst represents a strong barrier protecting the infective stages from tick immune mechanisms and mechanical pressure associated with tick molting [4]. The *Babesia* sporoblast lies freely inside the cytoplasm of tick salivary gland cells where it remains protected from constructional changes of internal organs accompanying tick molting. Moreover, there are potential analogies to the mechanisms of continuous transstadial as well as transovarial transmission: *Babesia microti* – the most investigated representative of ***Babesia* sensu lato** lineage – apparently lacks the ability of transovarial transmission and continuous persistence in the tick beyond one trophic stage of the tick vector [41].

Transovarial Transmission: A Unique Strategy of *Babesia*?

Transovarial transmission is considered as one of the most successful evolutionary strategies among members of the Apicomplexa. A single hard tick female lays down clutches of up to thousands of eggs [4]; this enables *Babesia* dissemination from a single infected tick individual to a legion of tick offspring. Although transovarial transmission is generally considered *Babesia*-specific, it has been described for *Babesia* sensu stricto only (Figures 2 and 3). Interestingly, haemogregarines of the genus *Karyolysus* form sporokinetes (analogous to *Babesia* kinetes) that invade ovarian cells of their mite vectors [42]. However, such an analogy evidently originates in the convergent evolution of the two distinct groups [34,43] (Box 2).

Transovarial transmission of *Babesia* is mediated through the invasion of kinetes to tick female ovaries (Figure 1). Establishment of the parasites inside tick ovarian cells is analogous to the process of invasion of the tick salivary glands when extensive kinete multiplication creates intracellular polymorphous networks. Newly generated kinetes persist within the cytoplasm of tick ovarian cells [44]. We would like to postulate that the developing tick embryo is later invaded by mature kinetes, which disseminate into the salivary glands. Mature sporozoites are subsequently produced and transmitted during the engorgement of the next tick generation. Apart from the essential role of vitellogenin [45], other molecular mechanisms associated with this process remain unknown.

Towards a Phylogeny-based Taxonomic Classification of Piroplasmids

Morphological and ultrastructural appearance, together with divergent host ranges and life cycle characteristics, resulted in the primary differentiation of three taxonomic genera of piroplasmids: *Babesia*, *Theileria,* and *Cytauxzoon* [5]. *Babesia* – currently defined as *Babesia* sensu stricto – has been distinguished from *Theileria* and *Cytauxzoon* by the absence of

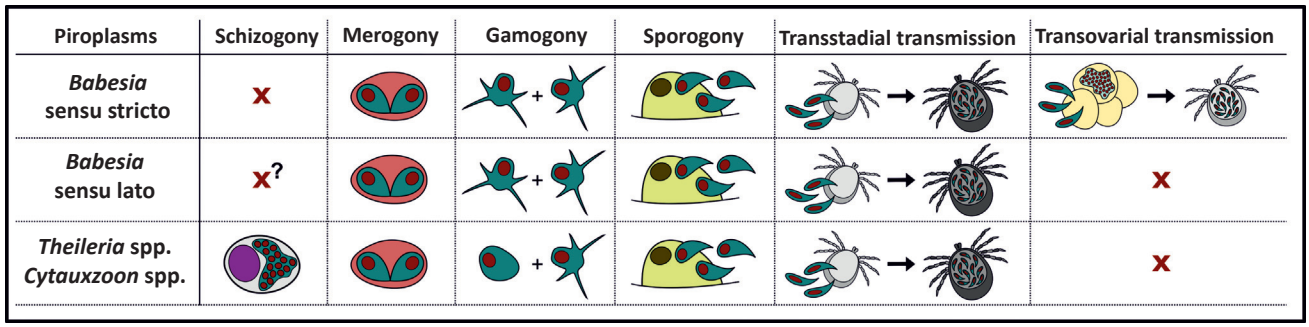

Figure 3. Morphological and Developmental Characters: Classic Taxonomy of Piroplasms. One of the main classification criteria distinguishing *Babesia* from *Theileria* and *Cytauxzoon* is the absence of schizogony. Although the schizont stage has originally been reported for the *Babesia* sensu lato species *B. microti*, it could neither be confirmed in this nor in other *Babesia* sensu lato species [47,48]. All piroplasmids undergo merogony, and in all piroplasmids gamogony takes place inside the tick midgut upon on-host feeding. The morphology of gametes is significantly different between *Babesia* versus *Theileria* and *Cytauxzoon*. *Babesia* gamogony is mediated by the fusion of two morphologically highly similar gametes known as 'ray bodies' [3], while *Theileria* and *Cytauxzoon* form morphologically distinguishable micro- and macrogametes, the archetypal gametes of the Apicomplexa. The arrest of sporogony and the formation of a sporoblast in the tick salivary glands is shown by all piroplasmids and enables successful transmission of piroplasmids via transstadial (horizontal) transmission. Exclusively, *Babesia* sensu stricto species demonstrate transovarial (vertical) transmission, allowing for a rapid dissemination of the parasite via infection of the next tick generation [4].

Box 2. Evolutionary History of Piroplasms

Since piroplasmids are characterized by an obligatory dixenous life cycle between an invertebrate and a vertebrate host, the coevolutionary processes of piroplasms with each host should be taken into account when contemplating their evolution. It is generally assumed that a tick ancestor is the evolutionary ancient host because closely related monoxenous apicomplexans *Cardiosporidium* and *Nephromyces* infecting invertebrate ascidians represent a sister group to piroplasmids [46]. This view is also supported by the occurrence of babesial key developmental phases (gamogony and sporogony) in the tick (= definitive host). A presumed piroplasmid-harbouring tick ancestor that entertained a scavenger lifestyle (like Holothyridae mites feeding on body fluids of dead organisms) might have adopted the blood-feeding tick lifestyle [89]. Thus, the obligatory dixenous life cycle – as it is characteristic for extent piroplasmids – most likely coincides with the evolutionary origin of the tick [46]. Tick species of the genus *Ixodes* are considered to have evolved around 217 mya (millions years ago), which corresponds with the appearance of mammalian stem clades around 235–205 mya [90]. As obligatory parasites, these ancient piroplasmid lineages may have gone extinct with their original protomammalian hosts (therapsids). However, as ticks are typically oligo- and/or polyphagous, their evolutionary survival (and that of their Piroplasmida parasites) has been ensured by them taking over alternative extant vertebrate lines as their principal hosts [91]. Monotremata (Prototheria) have already diverged from the ancestor of Marsupialia–Placentaria (Theria) at about 170 mya, whereas the marsupial–placental split has been estimated at about 150 mya. In contrast, the diversification of cenozoic modern mammals (Theria) only started around 60 mya. The outlined mammalian evolutionary splits would be expected to coincide with a coevolutionary diversification and speciation of piroplasmids and ticks [92]. Indeed, diversification into phylogenetic lineages of piroplasmids infecting monotremata, marsupials, and earliest mammalian groups (e.g., placental carnivores) corresponds with the outlined evolutionary diversification into mammalian groups, strongly suggesting cospeciation as a major evolutionary driving force.

schizont stages and the capacity for transovarial transmission (Figure 3). Piroplasmids that could not be assigned to any of the previous groups have been classified as *Babesia* sensu lato and are defined by the absence of both schizogony and transovarial transmission [46]. Although the presence of the schizont stage has been suggested for *B. microti* [32], it could never be subsequently confirmed. Moreover, schizonts have not been reported in any of the other *Babesia* sensu lato species [47,48].

Molecular Phylogeny of Piroplasms: Revealing Concealed Relations

Recent molecular phylogeny using the 18S RNA gene has completely changed the view on taxonomy and phylogenetic relationships within the Piroplasmida [46,49]. Piroplasmida

represent a polyphyletic assemblage comprising of at least six principal lineages, of which *Babesia* species represent at least three distinct clades [46]. Noteworthy, phylogenomic approaches confirmed the Piroplasmida phylogeny as initially demonstrated by 18S RNA gene comparisons [50,51]. However, although the genome of at least one species of six major piroplasmid lineages is known, the number of taxons with a sequenced genome is still too low to construct a comprehensive phylogenomic tree. In addition, phylogenomic approaches are expected to have the advantage of an enhanced phylogenetic signal but they need to be critically evaluated as they are complicated by the need for an unbiased selection of multiple suitable markers [52]. Nevertheless, the arrangement of six principal Piroplasmida lineages was confirmed by some alternative genomic approaches, for example, the comparison of paralogue expansions and profiles of **C1A cysteine-proteases** (or papain family of cysteine proteases) [53], deep-sequencing [54], and the comparison of mitochondrial genomes [55].

The present review builds on the above classification and integrates significant advances that have been achieved by molecular phylogeny of the 18S RNA gene, based on a significantly increased sample number [56]. As previously anticipated [46], recently sampled novel species and/or isolates segregated into novel clades [54,57–62]. Thus, the current picture on phylogenetic relationships of piroplasmids recognizes at least ten principal lineages (Figure 2 and Table 1) of which *Babesia* parasites constitute four clades: *Babesia* sensu stricto known as 'true' *Babesia*, and three lineages – Percei, Western, and *B. microti*-like groups – collectively referred to as *Babesia* sensu lato.

The updated phylogeny-based classification of the Piroplasmida is challenging the previous taxonomic classification. It reveals that *Babesia* and *Theileria* each represent a polyphyletic grouping, corroborating that phenotypic and/or life history characteristics are of limited value for a comprehensive taxonomy of the Piroplasmida [46]. Importantly, most lineages are faithfully associated within their respective vertebrate host taxon, suggesting that piroplasmid lineages have principally evolved by cospeciation with their mammalian and/or bird host taxons (Table 1). A notable exception constitutes the lineage *Babesia* sensu stricto (clade X), consisting of parasites of multiple and diverse mammalian and avian vertebrate hosts (Table 1).

The observed wide range of hosts indicates that multiple host switches between diverse mammalian and avian vertebrate groups have occurred independently. An intriguing question is: which features facilitated the more frequent vertebrate host switches of 'true' *Babesia* as compared to other piroplasmid lineages? As transovarial transmission is a trait exclusive to *Babesia* sensu stricto, it may account for an increased parasite–tick capacity to switch to novel unrelated vertebrate host families. However, host switches between vertebrate families/orders occur – although to a much lesser extent – in an additional two of altogether three *Babesia* sensu lato lineages (Western and *B. microti*-like group). The ability of *Babesia* sensu stricto to support transovarial transmission corresponds with their engagement to one-host ticks that persist on their vertebrate host during all tick developmental instars. *Babesia* sensu lato (and other piroplasmids) that lack the capacity for transovarial transmission, and transmit exclusively transstadially, require engaging two- or three-host ticks to ensure their successful dissemination to uninfected vertebrate hosts.

Babesia sensu lato can be clearly distinguished from the well-defined *Babesia* sensu stricto (Figure 3) and these organisms are represented by three different evolutionary lineages (Figure 2 and Table 1). The Percei group (clade V) includes exclusively *Babesia* species infecting birds. Species of the Western group (clade III) are known to infect several herbivores or carnivores, and humans as accidental hosts [6,63]. The *B. microti*-like group (clade I) branches into at least

Table 1. *Babesia*, *Theileria*, and *Cytauxzoon* Parasites and Their Common Designations, Molecular Phylogenies, Classical Taxonomies, and Diseases

Common designation		Phylogenetic		Classic taxonomy	Vertebrate hosts	Reference species	Diseases
		Clade[a]	Subcl[b]				
B. microti-like	*B. microti* group	I (I)	Ia	*Babesia* sensu lato	Rodentia, Macaca, (human)	*B. microti*	Human babesiosis
	B. vulpes group		Ib		Canidae, Mustelidae	*B. vulpes*	Canine babesiosis
	B. rodhaini		Ic		Rodentia	*B. rodhaini*	nk[d]
	B. felis group		Id		Felidae	*B. felis*	nk
Monotremata group		II		*Theileria* sensu lato	Monotremata	*T. ornythorhynchi*	nk
Western clade		III (II)		*Babesia* sensu lato	Bovidae, Cervidae, Canidae, Felidae, Herpestidae, Canidae, (human)	*B. duncani* *B. lengau* *B. conradae*	Human babesiosis, Canine babesiosis
Marsupialia group		IV		*Theileria* sensu lato	Marsupialia	*Theileria* spp.	nk
Peircei group		V		*Babesia* sensu lato	Aves	*B. peircei*, *B. poelea*, *B. ugwidiensis*	nk
Rhinocerotidae group		VI (IIIa)		*Theileria* sensu lato	Rhinocerotidae	*T. bicornis*	nk
Cytauxzoon		VII (IIIb)		*Cytauxzoon*	Felidae, Ursidae, Herspestidae	*C. felis, C. mauls*	Feline cytauxzoonosis
Equus group		VIII (IV)		*Theileria* sensu lato[c]	Equidae	*T. equi, T. haneyi*	Equine piroplasmosis
Theileria sensu stricto (true *Theileria*)		IX (V)		*Theileria* sensu stricto	Bovidae, Cervidae	*T. annulata* *T. parva* *T. lestoquardi* *T. orientalis*	Tropical theileriosis East Coast Fever Oriental theileriosis
Babesia sensu stricto (true *Babesia*)		X (VI)		*Babesia* sensu stricto	Marsupialia, Bovidae, Cervidae, Giraffidae, Rodentia, Canidae, Mustelidae, Ursidae, Felidae, Aves, (human)	*B. bovis* *B. bigemina* *B. canis* *B. ovis*	Bovine babesiosis Ovine babesiosis Canine babesiosis Human babesiosis

[a]Clade numbers correspond to phylogenetic lineages as assessed by 18S RNA gene phylogeny. In brackets, clades are given as previously defined in [46].
[b]Subl = subclade.
[c]*Theileria equi* has been originally described by classical taxonomy as *Theileria* sensu stricto [93].
[d]nk = not known.

four lineages that can be characterized based on molecular signatures, phylogeny, and host specificity (Table 1). Unfortunately, phenotypic and developmental characters, and even tick vectors, are unknown for almost all *Babesia* sensu lato. Of them, *B. microti* (clade Ia), a causative agent of human babesiosis [9], is the only parasite for which the life cycle has been described in detail [5,30,35,64]. *B. microti* features the smallest genome of the Apicomplexa, which is ~20% reduced as compared to the genome of *B. bovis* (*Babesia* sensu stricto) [50,65]. In addition, the *B. microti* genome contains ~7% fewer genes than the *B. bovis* genome and appears to lack the large multigene family coding for the VESA surface antigens of *B. bovis* [50]. Correspondingly, no gene homologues encoding proteins of the secretory pathways associated with spherical bodies of *B. bovis* are present in the *B. microti* genome [66], a feature that correlates with a highly reduced apical complex (Box 1) [64]. Hence, the evident and extensive phylogenetic distance of *B. microti* and *Babesia* sensu lato species from the 'true' *Babesia* is reflected in significantly reduced and different molecular characteristics requiring the development of distinct diagnostic tools and/or vaccines as compared to representatives of *Babesia* sensu stricto [67].

Therapeutic Targets: Future Perspectives

Applied research aimed at novel therapeutics against babesiosis is mainly focused on the blockage of *Babesia* merozoites. However, it might end up as a failing strategy because the intraerythrocytic development of *Babesia* is asynchronous and tightly connected to the sexual commitment of the parasite. Since *Babesia* gametocytes possess different antigens [20], there is a high chance that they would escape from the effect of therapeutic tools targeted to merozoites. In addition, description of *Babesia* gametocyte-specific antigens, including molecules involved in mechanisms governing parasite sexual commitment, might be a key to the development of transmission-blocking vaccines. Analogously, an effective strategy to reduce babesiosis might be based on selective blocking of *Babesia* sporozoites prior to their invasion of RBCs and establishment of merogony.

Despite the fact that the key parasitic developmental phases are localized to tick tissues (Figure 1), contrary to *Babesia* blood stages, knowledge of *Babesia* intra-tick development is sparse. Multiple schizogony steps appear during *Babesia* intra-tick progression – gamogony, intra-tissue propagation of kinetes and sporogony – and are, in principal, similar to schizogony of *Plasmodium* [68,69] or *Theileria* [70] taking place inside vertebrate host RBCs or leucocytes, respectively. Schizogony is an autonomous and remarkably different replication mode that cannot be explained by a generic eukaryotic cell model where cell-cycle progression is driven by the cooperation of protein **kinases** controlling ordinary synchronous division (recently summarized in [2,71]). However, studies of plasmodial schizogony revealed that the division of asynchronous nuclei is also governed by protein phosphorylation mechanisms. The essential and unique role of *P. falciparum* cdc2-related protein kinase 4 (*Pf*CRK4), among other functions, results in the unique initiation of multiple rounds of DNA replication during schizogony, as confirmed both by chemical inhibition [72] and destabilization domain conditional protein knock-downs [68]. Although kinases regulating nuclei division during *Babesia* schizogony have not yet been characterized, the analogy between *Babesia* intra-tick multiplication and *Plasmodium* schizogony could be due to *Babesia* and *Theileria* genome-encoded homologues of Aurora family kinases [73], known to play essential roles in eukaryotic cell division [74] and in mitotic control mechanisms during *Plasmodium* schizogony [73,75]. The reversible cell cycle arrest during transstadial and transovarial transmission is another important specific aspect of the *Babesia* life cycle. Although we can only hypothesize, the interruption of the *Babesia* cell cycle is presumably coordinated by protein phosphorylation since there are potential analogies to cell cycle arrest in *Plasmodium*, where re-entry into the cell cycle is induced by exogenous polyamines, and controlling mechanisms underlying this re-entry involve the expression of calcium-sensitive and mitotic kinases driven through specific transcription factors [76]. Apparently, the enormous role of kinases in the malaria life cycle, and their evolutionary conservation among plasmodial species [77], imply the necessity to describe the *Babesia* 'kinome' in order to fully understand cell arrest regulation in *Babesia* and to open avenues leading towards novel therapeutics based on the specific inhibition of *Babesia* kinases. Of interest are also other mechanisms of cell-arrest regulation, for example, the availability of extracellular isoleucine due to the absence of this amino acid in human haemoglobin [78].

Rapid biomass generation during schizogony in tick tissues is one of the most imposing events in the whole life cycle of *Babesia*. As in any other rapidly proliferating eukaryotic cell [79], babesial kinetes need to have perfected metabolic modes that efficiently convert glucose and specific amino acids into biomass and energy. By analogy to plasmodial intraerythrocytic schizogony [78,80], *Babesia* propagation in tick tissues exhibits a need for nutrients. The availability of most biochemical resources (glucose and glutamine, together with salvaged amino acids, fatty acids, and purines) is essential and likely complicated by the intracellular

localization of *Babesia* schizogony. Since *Babesia* (as in other organisms) generates biomass by aerobic glycolysis/fermentation and glutaminolysis in the presence of abundant glucose and glutamine, we propose a metabolic parallel between babesial schizogony and the Warburg effect, known from mammalian cancer cells (preference for fermentative glycolysis even under aerobic conditions) [80], with possible overlaps in molecular mechanisms and regulation.

Concluding Remarks

The life cycle of *Babesia* comprises specific strategies originating in the adaptation to blood parasitism and to the relatively complicated transmission by ticks (Figure 1). Research contributions dedicated to *Babesia* mainly investigate intraerythrocytic merozoites whereas other developmental stages, including those of intra-tick progression, remain largely neglected. We have proposed several events to be dissected at the molecular level using rapidly evolving tools of functional genomics and biological chemistry (reviewed in [81]). Detailed knowledge of particular molecular mechanisms involved in intra-tick development, dissemination, and transmission of *Babesia,* together with the above-described distant phylogenetic relationships between *Babesia* lineages, represent a fundamental basis for the design of effective therapeutic tools (see Outstanding Questions).

Outstanding Questions

Is the intra-tick propagation of *Babesia* (gamogony, intra-tissue propagation of kinetes and sporogony), in principal, analogous to the schizogony of *Plasmodium* and *Theileria*? What molecular regulatory mechanisms are involved?

To what extent do the surface proteins and secretory mechanisms of *Babesia* kinetes differ from those of the sporozoites?

What molecular mechanisms regulate sporoblast dormancy in the context of transstadial transmission?

How does the transovarial transmission of *Babesia* sensu stricto contribute to the parasitization of highly diverse vertebrate groups worldwide?

During transovarial transmission, how do parasites disseminate to the developing tick embryo and subsequent larvae? What molecular mechanisms are involved in this process?

Are generic therapeutic tools for *Babesia* possible regardless of the obvious molecular phylogenetic distances between *Babesia* lineages?

Are Piroplasmida–host and the tick–host specificities correlated due to host–parasite–vector coevolution?

How is the reduction of the babesial apical complex connected with the reduced persistence of the parasitophorous vacuole?

How does the apical complex structure correspond with the molecular phylogeny of piroplasmids?

What mechanisms does *Babesia* employ to evade the immunoprotective mechanisms of the host cell?

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