Characteristics, immunological events, and diagnostics of *Babesia* spp. infection, with emphasis on *Babesia canis*

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Abstract

Vector-borne infection constitutes a significant health issue in dogs worldwide. Recent reports point to an increasing number of canine vector-borne disease cases in European countries, including Poland. Canine babesiosis caused by various *Babesia* species is a protozoal tick-borne disease with worldwide distribution and significant veterinary importance. The development and application of molecular methods have increased our knowledge about canine babesiosis, its prevalence, and clinical and pathological aspects of the infection. Parasitologists and veterinary surgeons need an accurate description of the species responsible for canine babesiosis to improve diagnostic and therapeutic methods, as well as predictions for the course of the disease. Therefore, we decided to summarise recent knowledge concerning *Babesia* species and *B. canis*.

Keywords: babesiosis, *Babesia canis*, apicomplexa, genomics, gene expression.

Apicomplexa and its phylogeny. The phylum Apicomplexa (syn. apicomplexans) encompasses intracellular parasitic protists which frequently cause animal and human diseases. This group of organisms is cosmopolitan; previous research has found more than 5000 species, and the number is rising. The phylum is divided into two subphyla, Conoidasida and Aconoidasida, which are mainly distinguished by the occurrence and absence of conoid respectively. Taking a closer look at its taxonomy, the class Conoidasida contains two subclasses, Coccidia and Gregarinasina; the class Aconoidasida is also divided into two subclasses, Haemosporida and Piroplasmida (38). Despite the relatively keen interest of scientists in these organisms, a large number of the species have not been classified yet. The modern bioinformatic approach, based on protein analysis, offers a reliable phylogenetic analysis (Fig. 1) (59). The distinguishing feature of this protist is the occurrence of an endosymbiotic organelle – an apicoplast. It contains four membranes and is responsible for the long-term survival of the parasite (20). Similarly to other plastid organelles (chloroplast and mitochondria), the apicoplast contains its own circular genome but most of the protein-coding genes that build the organelle have been transferred to the nuclear genome (61). The organelle is considered to be involved in lipid metabolism (60). Another characteristic morphological structure of the phylum is the apical complex, made up of rhoptries, micronemes, apical polar rings, and conoids. In general, this structure is connected with host cell invasion including motility, adhesion, and formation of the parasitophorous vacuole (44). Invasion is physical when the host cell is penetrated by a parasite, which is unique among pathogens (51). The apicomplexans are characterised by a complex life cycle that may occur in one or two hosts. There are also different cell and host ranges within the phylum. *Toxoplasma gondii* can theoretically infect all nuclear cells in a wide range of hosts, while in contrast, parasites from the *Sarcocystis* spp. might be host specific (51). In general, the cell cycle of apicomplexans includes the following stages: 1) asexual replication, when the forms are sporozoite/merozoite, 2) gamogony, 3) sexual...
Fig. 1. Phylogenetic analysis based on Apicomplexan protein families. The number of protein families shared between at least two daughter taxa of a particular clade is circled at each node. On the terminal branches there is the number of species-specific protein families, where a family contains at least two proteins. Adopted and reproduced from Wasmuth et al. (59)

Fig. 2. Generic life cycle of Babesia spp. Sporozoites (Sz) are injected into a vertebrate host blood system. After invasion, Sz differentiate into trophozoites (T). Trophozoites undergo asexual division into two or four merozoites (M). Merozoites exit the red blood cells and invade new ones. Some groups of merozoites transform into gamonts or pregamonts (G). The process of gamogony and sporogony takes place in the tick. Gamonts ingested by a tick feeding on an infected host differentiate in the gut into gametes (called ray bodies or Strahlenkörper – (Sk)) that fuse forming a diploid zygote (Z, gamogony). Via meiosis division, zygotes give rise to motile haploid kinetes. After haploid kinetes multiply by sporogony, they penetrate the tick haemolymph and organs. The final stage of the development occurs in the salivary glands (Sg), where differentiation and multiplication occur. Kinetes transform into sporozoites that infect the vertebrate host after vector development into a subsequent life stage – larvae to nymph, nymph to adult (transstadial transmission, Ts). In large Babesia spp. kinetes also invade the tick ovaries and eggs, and infective sporozoites are formed in the salivary glands of the next generation larvae. This process is called transovarial transmission (To). Adopted and reproduced from Schnitter et al. (50)
replication, 4) ookinete, 5) meiosis, and 6) sporogony (53). With regard to the heterogeneity of the phylum, the genomics of the parasites are also variable. Selected features in this respect have been presented in Table 1. Babesia species are apicomplexan protozoa which parasitise two hosts: ticks and vertebrates, including humans. The first reports of babesiosis came from Texas (USA) in 1810. The parasite itself was detected in the 19th century by Victor Babes, and later called Babesia in honour of its discoverer (56). Babes found the parasite in erythrocytes of cattle, in which this pathogen is causative of severe infection worldwide. The main symptoms manifested by intermediate hosts (vertebrates) are fever, anaemia, and haemoglobinuria, potentially leading to death. This pathology is associated with the destruction of erythrocytes, host immune response, and organ dysfunction. Human babesiosis is caused by Babesia microti (North America), Babesia divergens (Europe) (29) or Babesia venatorum (China) (54). The distribution of the Babesia parasite among species is related to tick ecological range and may change along with climate change. In this review we concentrate on the molecular aspect of babesiosis and Babesia spp. biology. Since the prevalence of Babesia spp., which causes infections in dogs, is steadily increasing, and there is no effective vaccine against the illness, we resolved to analyse this issue.

Babesia life cycle. Babesia spp. multiplies in erythrocytes by asynchronous binary fission, resulting in a considerable pleomorphism. The replication eventually produces gametocytes that are ingested by ticks, i.e. invertebrate hosts/ vectors. The conjugation of gametocytes takes place in the tick gut, followed by multiplication via multiple fusion and migration to various tissues including the salivary glands, which are important organs in the life cycle of Babesia spp. Further development occurs in the salivary glands before transmission (28). Fig. 2 summarises the generic life cycle of Babesia spp.

Development in the invertebrate host. Ticks ingest Babesia-infected erythrocytes, but at this point most of the parasites are degenerated and destroyed. However, “pre-gametocytes” are not degenerated and they develop into gametocytes. Within several hours of gestation, elongated bodies characterised by an arrowhead-shaped ray begin to appear. The gametes fuse in the lumen of the tick digestive system. After zygote internalisation it transforms into a motile form – an ookinete. The ookinete exits in the midgut epithelium and invades the tick tissues and organs. The invasion of the ovary in female ticks results in infection of tick eggs. The developmental cycle of Babesia includes asexual multiplication, sporogony and the development of kinetes. Sporogony occurs in each tick developmental stage. Kinetes also invade the salivary glands of ticks where sporozoites are produced. These are passed into a mammalian host to close the cycle (50).

Development in the vertebrate host. Vertebrate hosts are infected by sporozoites from saliva after a tick bite. Babesia spp. sporozoites directly penetrate the red blood cells and there parasitic development occurs (50). Two merozoites are produced by binary fission. After erythrocytes are lysed, each merozoite invades a new erythrocyte and successive merogonies occur. The multiplication is asynchronous and various divisional stages of the parasite are present in the blood system at the same time. The location and size of merozoites differ between Babesia and host species. The size of merozoites determines the affiliation of Babesia spp. into large or small groups (Table 2) (28). In a large group Babesia spp. merozoites are larger than the erythrocyte radius, whereas in a small group the merozoites are smaller than it. This is not consistent with the genetic basis. Further, interspecific phenotypic variability, with different parasite sizes or forms in human, bovine, or rodent blood cells was described for B. divergens (50).

Host immune response to Babesia spp. All mammalian hosts are capable of becoming immune to Babesia spp., either after an episode of infection and subsequent recovery or as a result of vaccination. Humoral cell-mediated factors and nonspecific responses are involved in immunity to Babesia (Fig. 3). An infection with Babesia spp. triggers the production of antibodies. IgM and IgG1 and IgG2 are the isotypes specific for Babesia infections. The Babesia-specific antibodies resist the surface proteins of the erythrocytic stage and are involved in opsonisation of parasitised red blood cells.

Table 1. Genomic features of selected apicomplexan protozoans (1, 24, 25, 47)

<table>
<thead>
<tr>
<th>Genomic feature</th>
<th>C. parvum</th>
<th>P. falciparum</th>
<th>T. parva</th>
<th>T. gondii</th>
<th>N. caninum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Size (Mbp)</td>
<td>9.1</td>
<td>22.9</td>
<td>8.3</td>
<td>63.0</td>
<td>61.0</td>
</tr>
<tr>
<td>(GC) content</td>
<td>30</td>
<td>19.4</td>
<td>32.5</td>
<td>52.3</td>
<td>54.8</td>
</tr>
<tr>
<td>Number of genes</td>
<td>3807</td>
<td>5268</td>
<td>4035</td>
<td>7286</td>
<td>7121</td>
</tr>
<tr>
<td>Mean gene length</td>
<td>1795</td>
<td>2283</td>
<td>1407</td>
<td>2341</td>
<td>2553</td>
</tr>
<tr>
<td>Percentage of coding</td>
<td>75.3</td>
<td>52.6</td>
<td>68.4</td>
<td>28.3</td>
<td>29.7</td>
</tr>
</tbody>
</table>

bp – base pair; Mbp – megabasic pairs; GC – percentage of either guanine or cytosine in sequenced genomes.
Fig. 3. Theoretical model of the cells and molecules involved in the immunity to Babesia spp. (1). Establishment stage: IgG antibodies prevent erythrocytes by binding the free sporozoites. (2). Progression stage: cells of the innate immune system control the growth rate of the merozoites. The inhibition seems to rely on the production of soluble factors: IFN-γ by NK cells and TNF-α, nitric oxide (NO), and ROSs by macrophages (Mφ). (3) Resolution stage: T-cell lymphocytes (CD4+ IFN-γ) are responsible for parasite clearance. Adopted and reproduced from Homer et al. (29)

Table 2. Distribution, vectors, morphological and molecular characteristics of canine piroplasm species (modified from Irwin (30) and Matijatko et al. (42))

<table>
<thead>
<tr>
<th>Size</th>
<th>Species</th>
<th>Geographical distribution</th>
<th>Vectors in dogs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Large</td>
<td>B. canis</td>
<td>Europe, Asia</td>
<td>Dermacentor reticulatus</td>
</tr>
<tr>
<td></td>
<td>B. rossi</td>
<td>Africa, Nigeria, Sudan</td>
<td>Haemaphysalis elliptica (formerly H. leachi)</td>
</tr>
<tr>
<td></td>
<td>B. vogeli</td>
<td>Worldwide</td>
<td>Rhipicephalus sanguineus</td>
</tr>
<tr>
<td></td>
<td>Babesia spp.</td>
<td>North Carolina, USA</td>
<td>Unknown</td>
</tr>
<tr>
<td>Small</td>
<td>B. gibsoni</td>
<td>Worldwide</td>
<td>Rhipicephalus sanguineus?</td>
</tr>
<tr>
<td></td>
<td>B. conradae</td>
<td>California, USA</td>
<td>Rhipicephalus sanguineus?</td>
</tr>
<tr>
<td></td>
<td>B. microt-like (Theileria annae syn. B. vulpes)</td>
<td>Southern Europe</td>
<td>Most probably Ixodes hexagonus?</td>
</tr>
<tr>
<td></td>
<td>Theileria equi</td>
<td>Africa, Europe, Asia</td>
<td>Unknown</td>
</tr>
<tr>
<td></td>
<td>Theileria annulata</td>
<td>Africa, Europe, Asia</td>
<td>Unknown</td>
</tr>
</tbody>
</table>
Experiments conducted on equines and calves confirmed that the presence of IgG1 and IgG2 significantly reduces parasitaemia. Reduction of parasitaemia is caused by opsonisation and destruction of free parasites and infected erythrocytes by antibody-dependent cell cytotoxic mechanisms (34). Cell-mediated immunity was found to be involved in resistance to babesial infection with the recognition of the role of the spleen in defending the host against Babesia species (65). The spleen is populated by T cells, B cells, natural killer cells, and macrophages. Some of these cells are responsible for the protective function against Babesia spp. Experiments conducted on B. microti infection in rodents confirmed that T cells play a crucial role in resistance to babesiosis; T-cell mechanisms occur at the resolution stage. Nonspecific responses (innate immunity) have been found to play a role in the protection against babesiosis. In recent years, natural killer (NK) cells and macrophages were found to be important in antibabesial activity. Natural killers were studied in mice infected with B. microti, and a relationship between levels of NK cell activity and resistance to B. microti was found in mice in the early stages of infection (19). Macrophages were also found to play a protective role against Babesia infection. Macrophage depletion by means of silica eliminates protection against B. microti (70). Experiments performed on other Babesia spp. also demonstrated that macrophage inhibition or depletion disables protection against the parasite (29).

Genetic diversity. Comparative genome analyses between apicomplexan haemosporidian species have become quite common as large-scale and rapid sequencing technology has become more cost-effective. Knowledge obtained from cross-species or cross-genus comparisons may be helpful in improving existing prophylaxis, treatment, and management of the disease and may provide a better understanding of the underlying biology and evolution (37). The genetic heterogeneity of B. canis was first analysed using the chromosomal profiling of two laboratory strains, A and B (16). However, profiling has disadvantages as it is time-consuming and requires in vitro culturing of strains. PCR methods were reported to be useful for such analysis (16, 37). An analysis of the diversity of the Bc28.1 gene from B. canis isolates recovered in France showed that there are three genetically distinct B. canis groups (Bc-28A, Bc-28B, and Bc28-34) (10). A molecular analysis of the 18 sRNA gene from the Polish strains showed a genetic diversity between the analysed strains (2) and an association between the strain and virulence (3). The study conducted by Carcy et al. (10) on more than 200 isolates from Central Europe showed that only two genetic groups were prevalent (Bc28-A and -B), with higher prevalence of Bc28-A group. The analysis also showed that strains from genotype 34 were restricted only to France and there was a great variation in the distribution of strains belonging to genotypes A and B with respect to their longitudinal and latitudinal location in Europe.

Antigenic diversity and variation. Vaccination experiments showed that dogs vaccinated using soluble parasite antigens (SPA) of a B. canis strain were protected against a homologous challenge infection, but not against a homologous challenge infection with a heterologous B. canis strain B. This confirms that there are functional antigenic differences between B. canis strains (48). This antigenic diversity may be explained by allelic diversity. Different Babesia strains/clones express particular proteins from a particular family. This was shown by Carcy et al. (9) on MSA-1 and MSA-2 merozoite surface antigens. The antigenic diversity might be attributed to homologous recombination during sexual stages in the final host (tick); however, this process is still not well understood and some reports (i.e., 25), based on molecular analysis of gene organisation, point to the potential role of other mechanisms. Antigenic variation is generated by a complex phenomenon, a dynamic genetic process at the clonal level. This process has been reported in B. bovis and other protozoa parasites (4). This molecular event occurs when antigenic molecules are not expressed at the surface of infected erythrocytes. In this situation they may play a role in the specific adhesion of infected erythrocytes to the capillary endothelium of particular organs (42). This process is called sequestration and plays a crucial role in sustaining low-level chronic infection in immune animals. This helps to maintain immune responses (42). However, antigenic variation has not been shown for B. canis (42, 57).

Babesia genomics. According to the GOLD database, currently there are 17 genome projects dealing with Babesia spp. and five projects focusing on Theileria spp. The outcome of each project is unforeseeable because there are many correlations occurring during sequencing of the genome/transcriptome and many of the projects are still incomplete. Also some data have not been reported in the GOLD database (31). So far, the genomes of four Babesia species have been sequenced (7, 13, 15, 31). The first was B. bovis, the causative agent of acute babesiosis in cattle, followed by B. microti, a human pathogen. The extensive research by Jackson et al. (31) provides insight into B. divergens and B. bigemina. Additionally, some of the genomes have been resequenced. Selected genomic features of the sequenced Babesia spp. are presented in Table 3. A comparison of the genome size demonstrates the wide range in Babesia spp.: the genome of B. bigemina strain JG29 is twice as large as that of B. microti and its number of annotated genes is also bigger. A great divergence was also noted between the percentage of GC and TA nucleotides. Obviously, one should be conscious of the fact that the methodology of sequencing and bioinformatics has impact on the outcome. In resequencing B. divergens Rouen 1987 strain using Pacific Bioscience long-read sequencing,
Cuesta et al. (15) obtained a 2.5 Mb longer assembly, which included a large number of repetitive sequences. However, simultaneously using different annotation methodology for resequencing they obtained over 400 genes fewer than Jackson et al. (31). So far, there is no gold standard in NGS methodology but the necessary techniques are constantly being developed. Generally, the genomic analysis of *B. microti* has revealed that the parasite is a member of a distinct apicomplexa family. *B. microti* differs also from other Babesia species by featuring short 20–25 bp introns. This fact might be connected with the size of the *B. microti* genome. For *B. microti*, the apicoplast genome was also sequenced yielding a size of 28.7 kb (circular). The annotation analysis showed the genome of this organelle encoding the transcription and translation events (connected with apicoplast metabolism), structural and regulatory proteins, and enzymes harbouring an apicoplast targeting motif. The mitochondrial genome of *B. microti* is linear and 11 kbp in size (13). In *B. bigemina*, the larger number of coding sequences in comparison with other species is connected with unique gene duplications of conserved gene families and with a greater number of species-specific sequences encoding hypothetical proteins. The gene composition of *B. bovis*, *B. bigemina*, and *B. divergens* showed the following values: 67.6% to 81.2% of genes are conserved between species, 6.6% to 10.9% of genes are semi-conserved, and 12.2% to 21.5% of genes are unique for each parasite. A comparison of the *B. bovis* genome with genomes of the parasite belonging to various apicomplexa families demonstrates the closest relationship with Theileria sp., which also corresponds to the phenotypic futures of the parasites. However, major differences were found in relation to the Plasmodium genomes, which represent the same type of infection. In the context of other Babesia species, the major genomic features support this claim. The untranslated region (5’ UTR) of *B. bovis* is estimated to be 152 bp. The analysis of the genomes of attenuated and virulent strains demonstrated the reduction of genome diversity in the attenuated strain as well as highlighted the significant single nucleotide polymorphism among 14 genes (37). Pedroni et al. (46) managed to sequence the transcriptomes of virulent and attenuated *B. bovis* strains. According to microarray data, 2896 to 3293 genes were detected. In the virulent strain around 100 additional transcripts were found. The proportion of coding sequences corresponds to the number of genes and in this particular study ranged between 78.3% and 89%. In comparison to the other two strains, 749 to 773 genes were differently expressed in *B. bovis*.

### Diagnostic methods

**Clinical practice.** Regardless of modern approaches to parasite diagnosing, clinicians still rely on general clinical examination when dealing with a Babesia spp. infection. The clinical picture of babesiosis varies depending on the etiological agent and its host. The course of infection is multifactorial and connected with the extent of infection and the immunological condition of the host. In case of canine babesiosis, one should suspect it where symptoms present such as lethargy, anorexia, elevated temperature, colour change in mucous membranes and urine, abnormal urine composition, renal failure, icterus, hypotension, acute respiratory distress syndrome, enlarged spleen, vomiting, diarrhoea, weakness, tachycardia, or tachypnea (52). More detailed examinations have shown that an experimental infection with *B. canis* resulted in transient low
parasitaemia (1%–2%), a decrease in haematocrit, thrombocytopenia, an increase in the activated partial thromboplastin time (APTT), and hypotension (49).

**Microscopic methods.** The taxonomy evaluation of *Babesia* spp. has been changed in parallel with improvement of diagnostic methods. Historically, the method of choice was based on blood smear analysis. Initially researchers were able to distinguish piroplasms and Haemoproteus spp. The parasite shape and morphology were considered in diagnostics, including various features of each stage of the life cycle (56). Technically, we can distinguish two types of blood slides used in *Babesia* spp. diagnostics – thin and thick films. The basic method of staining is Giemsa and its modifications. It enables distinctions to be made across the Piroplasmida order and some basic species to be recognised. Alternatively, diagnostics can be performed using acridine orange staining. With this method, the parasite is easier to find; however, it is impossible to recognise various morphological features and interspecies differentiation cannot be made. There were also attempts to use the quantitative buffy coat (QBC) system in *Babesia* diagnostics (56). According to their morphometric features, canine *Babesia* spp. can be split into small and large *Babesia* groups, with sizes of 0.3 × 3 μm and 2 × 6 μm respectively (52). Blood parameters during the course of babesiosis in dogs are specific. The diagnostician should pay attention to thrombocytopenia, leukopenia, lymphopenia, and neutropenia. Low values of red blood cell count, packed cell volume, and haemoglobin were noted (32).

**Serological methods and flow cytometry.** Along with the development of serological methods, various applications have been proposed for *Babesia* spp. diagnostics including indirect haemagglutination (27), complement fixation test (40), latex bead agglutination (58), rapid card agglutination (57), indirect immunoﬂuorescence (23), ELISA (23), and cELISA (26). Given the signiﬁcance of public health and animal breeding, most of the assays have been developed to diagnose human and bovine babesiosis. However, some serological tests have been developed for canine babesiosis (35). Immunochromatographic tests (ICT) have also been developed (58). The serodiagnostic method is convenient, efficient, and suitable but there are numerous reports of cross-reactivity between various *Babesia* spp. species, non-speciﬁcity of results, and dissociation between results obtained with various serological methods (23). Cell staining with hydroethidine and SYTO 16 gives the opportunity to improve the performance of diagnostics by using flow cytometry (62). Recently, the label-free technique, dielectrophoretic sorting, or impedance spectroscopy on a microfabricated flow cytometer have been applied for enumeration and sorting of *B. bovis* (45).

**Molecular diagnostics.** Molecular diagnosis, especially the development of PCR, has revolutionised microbial diagnostics. The same can be said of *Babesia* spp. diagnostics. The gene encoding the small subunit ribosomal RNA (ssu-rDNA) has been widely used for diagnosis, differentiation, and to reveal the phylogenetic relationships among the apicomplexans (18), as well as *Babesia* spp. using either PCR or Southern blot methods (18). Similar to other protozoans, the genetic marker first utilised was the SSU-rRNA gene. This is a well-characterised marker, which is relatively conservative at the species level. The obtained sequences conﬁrmed previous studies that highlighted the occurrence of three distinct species of *B. canis: B. canis canis, B. canis rossi, and B. canis vogeli*. Previously, they were considered a single *B. canis* species, only distinguishable from *B. gibsoni*. What is more, a phylogenetic analysis demonstrated the distinctness of *B. canis vogeli* from the other two species of *B. canis*. For convenience, restriction fragment length polymorphism (RFLP) assays have also been developed (11). Over the years, methods based on amplification of various fragments of the ribosomal RNA gene have been proposed (41). The improved methods now include the semi-nested, nested, multiplex, and touch-down PCR approaches, which might have a positive impact on the sensitivity of diagnosis of isolates from animal blood. The SSU-rRNA gene has also been applied in diagnostics of other piroplasms species occurring in dogs, including *T. annae* (syn. *B. vulpes*) (6) and *B. conradae* (33). Technical improvements allow high-resolution melting curve quantitative Förster resonance energy transfer (FRET)-PCRs to be designed. Because the results are quantitative, the method can be applied in canine babesiosis infection level monitoring and it increases sensitivity up to seven copies of the *Babesia* spp. 18SrRNA gene per millilitre of whole blood. No risk of contamination occurs during post-PCR steps. A molecular diagnostic method that uses a single marker can be uncertain and needs to be conﬁrmed. This was demonstrated with regard to other parasitic protozoans. For babesiosis diagnostics, several molecular markers have been proposed including the P29 gene (22), rhoptry-associated protein 1 (RAP-1) (64), β-tubulin (8), heat shock proteins (HSP-70) (63), and the Bc28.1 gene (10). It should be emphasised that molecular methods are highly sensitive and specific, which is important in the context of subclinical and/or low parasitaemia. Comparison of blood smears, flow cytometry, duplex PCR, and duplex nested PCR (nPCR) have shown the advantage of duplex nPCR in terms of specificity and sensitivity (14). Recent studies conducted in Slovakia also affirm the reliability of PCR in comparison with microscopy and serology (indirect ﬂuorescent antibody technique (IFAT)) (36).

**Vaccines.** Most research has been focused on bovine babesiosis, because it is a serious livestock health concern. The ﬁrst attempts to develop a vaccine used an attenuated organism passaged in vivo in calves. The vaccine obtained proved to be a successful reducer of virulence (12). Although the vaccine was produced
using good manufacturing practices and has become adequately standardised, it has several drawbacks including: potential adverse reactions, handling or storage issues, antagonistic and synergistic interaction with concurrent disease, stress, and inadequate immune response (17). For example, the vaccine developed in Cuba was found to have a 2% rate of post-vaccination incidents (5). Another strategy for building adequate immunity to Babesia parasites is the subunit vaccine. Numerous proteins have been put forward as vaccine candidates, e.g. SBP-1, SBP-2, SBP-3, high molecular weight (MW) antigen I, RAP-1, 12D3, MSA-1, MSA-2, HSP family proteins, and Babesia divergens apical membrane antigen-1 (BdAMA-1) (43). These vaccines are very promising, because they are safe, high-throughput, eminently manufacturable, and putatively efficient. However, due to the changeable nature of this target, the design of the vaccine is complex (65), and in practice the solution obtained may not induce immunity to different strains. With respect to canine babesiosis control, two commercial vaccines are available in Europe now (48). They are based on soluble antigens derived from B. canis and B. rossi cultures. However, their efficacy can be questioned because they do not prevent infection and only affect the course of the disease. Immunity lasts only up to six months and a skin reaction at the injection site has been noted (21). Thus, vaccination does not stop the spread of B. canis. The efficacy of the vaccine is even lower in the control of B. rossi infection. Currently, there is no available vaccine against canine babesiosis caused by small Bbabesia species. Recently, Sunaga et al. (55) tested soluble antigens from an attenuated B. gibsoni culture for vaccine preparation. The challenge showed a reduction of parasitaemia and partial protection against infection. However, the research was carried out on a small number of animals, and now it is essential to perform proper clinical trials of the vaccine prior to its implementation. Vaccine development still poses a huge challenge to scientists worldwide, and widespread infections are still a serious issue.

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