Babesiosis as a disease of people and dogs. Molecular diagnostics: a review

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ABSTRACT: *Babesia* is the causative agent of babesiosis, a tick-borne zoonosis which has been increasingly described throughout the world. *Babesia microti* and *Babesia divergens* are the etiological agents of human babesiosis. *Babesia canis* is the principal etiological agent of canine babesiosis. Currently, the diagnostics of babesiosis is based mainly on serological methods and the immunofluorescent antibody test (IFA) is most commonly used. However, even in the acute phase of the disease, seroconversion does not always occur. Clinical symptoms, because of their unspecificity, cannot be used to make a correct diagnosis. In this situation other diagnostic methods are needed. The use of PCR (polymerase chain reaction) is the most promising of these. An advantage of this method is that it allows identification of the parasite in the early stage of disease which enables early diagnosis, implementation of therapy and avoidance of complications. However, the standardization of this technique remains to be carried out. Selection of a genetic marker for PCR is very important for the sensitivity of this technique and it is discussed in this paper.

Keywords: babesiosis in dogs and humans; diagnostics; PCR; selection of markers to PCR

Contents

1. Introduction

Babesiosis is a dangerous, invasive disease of humans and animals. Probably the first described case of an epidemic caused by the *Babesia* genus was a cattle mortality described in the biblical Book of Exodus (Homer et al., 2000). In 1888, Victor Babes described intraerythrocytic microorganisms responsible for the death of 50 thousand cattle in Romania and classified them as Bacteria. In 1893, Kilborne and Smith described a factor of Texas cattle fever, giving them the rank of genus and name *Babesia* as classifying them as Protozoans (Kjemtrup and Conrad, 2000).

2. Etiological agents of human and canine babesiosis

In general, two species, *B. microti* and *B. divergens* are etiological agents of human babesiosis. The first is a North American species, a parasite of rodents responsible for the majority of diagnosed cases. In Europe, the major etiological agent is *B. divergens*, a cattle parasite. However, recent studies have also revealed the presence of *B. microti* in Europe. Studies in Poland focus mainly on *B. microti* reservoirs, but the assessment of distribution of both protozoan species in the tick *Ixodes ricinus* have also been undertaken. They

have shown that both *B. divergens* and *B. microti* are present in these ticks and that they are a potential danger for human health. The presence of the latter one, in particular, makes a strong argument against the artificial division of pathogenic species into North American and European groups.

Despite the European distribution of *B. microti*, there are no confirmed cases of human babesiosis caused by the European strain of this species. This can be explained by the fact that the main vector of this pathogen is *Ixodes trianguliceps*, a tick which infests rodents (Homer et al., 2000; Kjemtrup and Conrad, 2000). The studies of Walter and Weber (1981) indicate that at least a few strains of *B. microti* may be transmitted by *I. ricinus.* That has been recently confirmed by the experimental transmission of pathogens (strain HK) from gerbils to larvae and nymphs of *I. ricinus*, in which the DNA of protozoans was detected with the PCR technique in metamorphosed nymphs and adults. Some infected nymphs were placed close to non-infected gerbils and left to parasitize. Protozoans were detected in blood smears from 12 to 17 days after infestation. It is interesting that the American strain G1, differing significantly from strain HK in the gerbils' erythrocytes turned out to be pathogenic for *I. ricinus* after repeated exposure. Therefore, it is highly probable that the remaining European *B. microti* strains may also infect *I. ricinus* and *B. microti* may potentially cause zoonosis in many areas of Europe.

In 2006, Casati et al. (2006) investigated the presence of *Babesia* in 1 159 specimens of *I. ricinus* collected from four forest areas in Switzerland. They obtained sequences of gene fragments encoding 18S rRNA for the small ribosomal subunit, characteristic for three species pathogenic for humans: *B. microti, B. divergens* and *Babesia* sp. EU1.

In our earlier studies in Poland, we revealed the presence of *Babesia* species in *I. ricinus* collected in Northern Poland by using PCR (Skotarczak et al., 2002, 2003). By PCR-RFLP analysis, we also obtained products that were characteristic for both species (Sawczuk et al., 2005). There are more and more doubts being expressed concerning the relationship between species occurring in the USA and those in Europe; therefore, we have sequenced the gene encoding 18S rRNA from isolates coming from the same ticks. From 26 samples, we obtained similar sequences, which were highly homologous to those deposited in the GenBank, belonging to *B. divergens* and from two samples, sequences belonging to *B. microti* occurring in Europe, Asia and North America (Pieniazek et al., 2006). The construction of trees and phylogenetic analysis carried out on the basis of obtained 18S rRNA gene sequences have shown that sequences identified as *B. divergens* and *B. microti* create a common clad with the European, and not American, sequences received from the GenBank.

Furthermore, recent studies on samples coming from patients with borreliosis suggest that coinfection of *Borrelia burgdorferi* and *B. microti* is possible, and that, as described in our earlier studies or in those from Switzerland, the same is true for *I. ricinus* (Casati et al., 2006).

Piroplasmosis in dogs is acknowledged as being an emerging disease. It is widely spread all over the world and is caused by several *Babesia* species. It has been known for several years that a species similar to *B. microti*, described as *B. microti*-like, may be the etiological factor of babesiosis in dogs (Camacho et al., 2001). However, babesiosis in dogs is caused mainly by *Babesia canis*, which has turned out to be a collective species, represented by three subspecies (*B. canis canis, B. canis rossi* and *B. canis vogeli*), whose vectors are ticks not belonging to the *Ixodes* genus. Vectors for the above mentioned subspecies are *Dermacentor reticulatus* (*B. canis canis*), *Haemaphysalis leachi* (*B. canis rossi*) and *Rhipicephalus sanguineus* (*B. canis vogeli*). *B. canis* is distributed in southern Europe, North America, Africa and Asia (Okinawa). Additionally, the pathogenic species *Babesia gibsoni* was found in dogs in Africa, Asia, America, Australia (Brown et al., 2006) and in Europe (only as dispersion), often together with *B. canis*. Studies of DNA isolates from the blood of dogs carried out in Spain (Criado-Fornelio et al., 2003) revealed that *B. canis vogeli, B. canis canis, Babesia equi* and *Theileria annae* were present among the Piroplasmida.

3. Babesiosis – occurrence and the clinical picture

Most incidents are connected with the seasonal activity of ticks. In Poland there are two peak activity periods: during spring-summer (May to June), and summer-autumn (August to September). The risk of tick infestation is increased during this time and correlates with a higher risk of the pathogen's transmission.

Babesiosis is more often observed in elderly people (those around the age of 60), individuals who have recently undergone a splenectomy, or those with immunodeficiency (HIV). However, most incidents of babesiosis remain undetected and undiagnosed, especially in people with a correctly functioning immune system. They constitute a potential danger if they donate blood. 40 incidents of transfusion babesiosis have been documented in the USA, in which the blood donor was a symptomless carrier (Kjemtrup and Conrad, 2000). A very low level of parasitemia, even up to 10 months after, is detected in those people.

Most diagnosed cases of human babesiosis have occured in the USA. In New York state alone, in the period 1982–1991, 136 people fell ill (Meldrum et al., 1992). Every year, several dozens of new cases are diagnosed. 29 infections caused mainly by *B. divergens* have been reported in Europe so far. But it is assumed that the scale of problem is much larger and that the disease is underdiagnosed. Most European cases have been described in France (10), six were observed in the British Isles and also others have been documented in Spain, Sweden, Switzerland, Belgium, the former Yugoslavia and others (Gorenflot et al., 1998; Homer et al., 2000).

Symptoms of babesiosis in dogs include fever, lack of appetite, apathy, haemoglobinuria, bilirubinuria, polichromasia, progressive haemolytic anemia, spleeno and hepatomegalia, jaundice, vomiting and death. Babesiosis caused by *B. canis rossi* is often lethal in spite of therapy; *B. canis vogeli* leads to a mild, often asymptomatic form of babesiosis, while *B. canis canis* causes babesiosis with an intensity within the range mentioned above.

Infections of dogs with *Babesia* piroplasms have been documented all around the world (Ano et al., 2001; Schaarschmidt et al., 2006). Brown et al. (2006) investigated 215 isolates of Australian dogs' blood. They detected the DNA of *Anaplasma platys* (69/32%), the DNA of *B. canis vogeli* (22/10%), and of both pathogens together in 24 cases (11%). In Poland, infections of dogs caused by *B. canis* protozoans were first reported in the 1960s. Babesiosis in dogs occurs mainly in the northern-east and eastern part of Poland, especially in Lubelska Upland and is directly connected with the occurrence of the competent host, *Dermacentor reticulatus*, which is also a vector for *B. canis*. Its absence in the northern-west and western part of Poland, therefore, prevents the spread of *B. canis* in this part of Poland. Several hundred cases of babesiosis in dogs in the endemic areas of eastern Poland are reported every year. According to data from literature, in Lubelska Upland, about 300–400 cases of this illness were reported in 1995–1997. In Warsaw, 430 cases were noted in the period of time from 1992 to 2002, and in the year 1997 alone babesiosis was the cause of about 50 moratlities in the dogs of the city (Kotomski, 2002; Gorski et al., 2004).

4. Diagnostics

The most common technique of diagnosing babesiosis involves taking smears from full blood and staining according to the Giemsa or Wright method. Oval, pear- or ring-shaped and amoeba-like forms of the parasite can be seen in erythrocytes. Sometimes, in cases of low parasitemia, infected cells can remain undetected. *Babesia* species cannot be identified on the basis of morphology and it is difficult to distinguish between *Babesia* and *Plasmodium* parasites.

The inoculation of laboratory animals with blood from presumed cases of babesiosis is time consuming because the first symptoms occur after 10 days. The parasitemia level increases, and blood smears, therefore, may help in the identification of pathogen. Immunological and serological methods are characterized by their high specificity and sensitivity. The IFA test is one of the most common tools used in the diagnostics of babesiosis. This method involves the binding antibodies from the serum of infected individual with a labelled antibody for IFAT. It is appropriate for monitoring infection and in chronic cases of the disease. Sometimes antibodies can still be detected years after recovery. However, when attempting to make an early diagnosis in the initial phase of the disease, i.e., when serum is sampled before the production of antibodies, a false negative result may occur. Patients who have recently undergone a splenectomy or HIV positive individuals may possess very low, undetectable levels of antibodies. Schaarschmidt et al. (2006) showed that in seven dogs with clinical symptoms of babesiosis, only two had specific antibodies and in only two were trophozoites of *Babesia* in blood smears detected. However, each was PCR positive. These and other studies show that another diagnostic method is essential (Schaarschmidt et al., 2006).

4.1. Molecular diagnostics: selection of a genetic marker for PCR and the sensitivity of this technique

One of the most frequently used tools in the detection of *Babesia* protozoans is PCR or PCR-RFLP. The selection of appropriate genetic markers to detect *Babesia* DNA is extremely important. Unlike most pathogens transmitted by ticks, such as *B. burgdorferi*, the genome of *Babesia* has not been completely sequenced. From the established pool of genetic markers used in detecting these and many other protozoans, the most commonly used are fragments of genes encoding rRNA for the small ribosomal subunit, because they are present in the genome of every eukaryotic organism. There probably exist three combinations of genes encoding the ribosomal RNA in *Babesia* genome. They are present in the following order: 18S-ITS1-5,8S-ITS2-28S. The 18S rDNA gene (18S rDNA, ssu rDNA) encoding rRNA of the small ribosomal subunit is one of the most frequently used molecular aims in the diagnostic and epidemiological studies of protozoans of the *Babesia* genus. The size of this gene is different in different species and contains between 1 720 and 1 770 bp. There exist conserved sequences, i.e., with sequences of nucleotides identical in most or in all *Babesia* species and also in species closely related to them. Such an organisation of the gene enables the designing of primers complementary to the conserved sites in PCR and the detection of the DNA of a large group of related organisms. The identification of different *Babesia* species will be possible only if the obtained product is digested with restriction enzymes. Another possible way to differentiate species is the nested PCR method, because the gene encoding 18S rRNA also includes variable sites. In *Babesia*, as well as in other *Eukaryota* the gene harbours eight variable regions numbered V1 to V5 and V7 to V 9 (region V6 occurs in *Prokaryota*). The biggest and the most changeable region is the fragment of the V4 gene comprised of a sequence of 300 bp. Designing primers for nested PCR, complementary to genes unique in terms of their sequences enables the amplification of products characteristic for some *Babesia* species or even strains within this species. Furthermore, a total sequencing of this gene, with its conserved flanking regions bookending the variable interior will, after amplification and sequencing, enables comparison with sequences already existing in GenBank and the precise identification of different species (Zahler et al., 2000).

Persing et al. (1992) were the first to use PCR for the diagnostics of human babesiosis. Krause et al. (1996) conducted a blinded study of the sensitivity and specificity of the PCR-based test with primers B1 and Bab4 complementary to the gene fragment (238 bp) encoding the 18S rRNA of *B. microti*. They examined patients with babesiosis and an asymptomatic group residing in a region in southern New England (USA) where babesiosis is enzootic. They found that PCR was as sensitive and specific as the use of Giemsa-stained blood smears and inoculation of hamsters.

In another study where PCR was applied as the method of diagnostics of babesiosis, Aktas et al. (2005) used a pair of primers complementary to the sequence of the gene encoding the ss rRNA of *B. ovis* isolated from sheep in eastern Turkey. They obtained a product characteristic only for this species. In order to assess the sensitivity of PCR, they used several dilutions of DNA samples (from 10^{-1} to 10^{-9}). The PCR was sensitive enough to detect parasite DNA from a dilution of 10^{-5}) with 0.00001% parasitemia. Moreover, at the same time they carried out observations of blood smears under light microscopy and only four samples were positive for DNA of *B. ovis* whereas in PCR 21 samples were shown to be positive. The authors concluded that PCR can significantly simplify the diagnostics of babesiosis when the infectious factor is not evident or when serological tests are falsely negative.

With the aim of developing the molecular diagnostics of babesiosis, Birkenheuer et al. (2003) carried out semi-nested PCR to detect and differentiate the DNA of *B. gibsoni* (Asian genotype), *B. canis* subsp. *vogeli*, *B. canis* subsp. *canis*, and *B. canis* subsp. *rossi* in canine blood samples in the USA. They designed pairs of primers to amplify an approximately 340-bp fragment of the 18S rRNA genes from *B. gibsoni* (Asian genotype), *B. canis* subsp. *vogeli*, *B. canis* subsp. *rossi*, and *B. canis* subsp. *canis* but not mammalian DNA. The authors emphasized that in the diagnostics of babesiosis, the determination of the species, subspecies and even genotype that caused the babesiosis in dogs is very essential, because virulence, prognosis and response to medicines against *Babesia* may be different in every organism.

Ano et al. (2001) tested the sensitivity of the nested PCR protocol carried out on the basis of

the gene fragment encoding 18S rRNA in dogs experimentally infected and in naturally infected patients. They found that visualization of the product after the first-round of PCR was poor in both groups and that only after the second round was there a clear band on the agarose gel. The authors checked the sensitivity of the described nested PCR protocol by using different dilutions of blood samples and found that the result was positive at 0.0001% parasitemia.

The sequences of the 18S rDNA gene have also been used to differentiate species of piroplasms occurring in dogs with RFLP (Restriction Fragments Length Polymorphism) protocols (Jeffries et al., 2003). The enzyme used for the restriction of the nested PCR product which was carried out to intensify the signal has allowed the differentiation of *Theileria annae, T. equi, Babesia conradae, B. gibsoni, Babesia* sp. (Coco) from each other and from the *B. canis* subspecies. Other species, not only those occurring in dogs, may also be differentiated by PCR. The authors carried out a sensitivity test for the discussed PCR-RFLP protocol and found out it to be high, as it was possible to use it at a parasitemia level of 2.7×10^{-7}% (when the amount of DNA template is smaller than 1,2 molecules when DNA is isolated from full blood collected into EDTA). Moreover, the application of a filter paper to blood samples, significantly enhances the detection of piroplasms and according to authors, the described protocol may be used for standardizing a routine screening of piroplasms in dogs.

Furthermore, sequences of the 18S rRNA gene for small ribosomal subunit, obtained from DNA isolates from the blood of dogs living in southern Europe (Spain) were used by Criado-Fornelio et al. (2003) for phylogenetic analysis. The complete gene sequences for *B. ovis* and *B. bovis* showed only 95% homology with those previously placed in the GenBank. A phylogenetic tree constructed on the basis of their own sequences and 44 collected from the GenBank divided the piroplasmids in five clads. The first is the *B. microti* group, with *B. rodhaini*, *B. felis*, *B. leo*, *B. microti* and *T. annae* (proposed name for the group, without taxonomic value: Archaeopiroplasmida). The second is Western USA *Theileria*-like group (proposed name: Prototheilerida). The third: *Theileria* group, containing all *Theileria* species from Bovinae (proposed name: Theilerida). The fourth group includes *B. canis* and *B. gibsoni* from dogs together with *B. divergens* and *B. odocoilei* (proposed name: Babesida), and the fifth is composed mainly by *B. caballi*, *B. bigemina*, *B. ovis*, *B. bovis* and *Babesia* sp. from cow (proposed name: Ungulibabesida). The bootstrap support obtained with the several analytical procedures for this new dichotomy of Babesidae was very high and so the reliability and accuracy of the constructed tree were also high.

Another molecular marker that allows the precise identification of *Babesia* species is the gene encoding the β-tubulin protein (component of microtubules). This gene occurs in the cells of all organisms. Studies have shown that in this species, the β-tubulin gene is approximately 1 350 bp in length and that it encodes a protein whose size is 440 amino acids. There are two introns within this gene and the first one exhibits large variability in terms of length as well in its nucleotide sequence (Caccio et al., 2000). The method is based on the specific amplification of this variable fragment and uses degenerate primers complementary to the conserved sites of this gene. The PCR products are of different length depending on the *Babesia* species (from 310 bp in *B. microti* to 460 bp in *B. caballi*); their length is determined by the presence of the intron which varies in length (Caccio et al., 2000). Identification of the species may be carried out through direct analysis of the length of amplicons after PCR, the product together with internal primers (nested PCR), or by using the RFLP protocol. The RFLP procedure, which uses restriction enzymes, allows the generation of a band pattern characteristic for only one *Babesia* species (Caccio et al., 2000).

Other gene sequences may be used as the molecular target for DNA detection and for differentiating *Babesia* species are genes encoding the Heat Shock Proteins (HSP 70). The HSP proteins are a group of proteins which are activated and biosynthesised during different kinds of cell stress and they occur in all living organisms (Yamasaki et al., 2007). Their role lies in the regulation of the vital functions of cells, for example, the controlling of cell divisions by connecting cells in complexes together with other cell proteins. A main polypeptide of this family is the HSP 70 protein – the most conserved and most commonly occurring one among all of these proteins. Among the protozoans of the *Babesia* genus, a sequence of gene encoding this protein has been so far described in several species, i.e., in *B. microti, B. bovis, B. rodhani* and *B. gibsoni.* Among these species, the length of this sequence that encodes

a protein of about 650 amino acids varies slightly and is in the region of about 1 940 bp. It is already known that in *B. microti* only one copy of the *hsp70* gene is present, whereas species belonging to *Plasmodium* have at least 5–6 copies (Yamasaki et al., 2007). The *hsp70* gene shows a large conservatism in its nucleotide sequence even among non-related organisms. Therefore, this method based on the amplification of a whole gene or its fragments finds its application mainly in molecular phylogenetic analysis.

5. Summary

Traditional methods have been complemented or even ousted by the molecular ones, of which the polymerase chain reaction (PCR) is the best technique. However, the standardization of this technique remains to be carried out. Different molecular targets were tested for PCR and genes encoding the rRNA of the small ribosomal subunit were found to be the most useful. Within the ribosome, regions conserved in evolution can be distinguished, i.e. having the nucleotide sequences similar to the majority or all *Babesia* species and to others closely related to them. Such an organisation of the gene enables the design of primers complementary to conserved sites for PCR and which can detect a large group of related organisms. Another molecular marker allowing the accurate identification of *Babesia* is the gene encoding the β-tubulin protein. There are two introns within this gene; the first one exhibits much variabilty with regard to length as well as to the nucleotide sequence. Therefore, the PCR products are of varied lengths depending on the *Babesia* species. However, these differences are too small for the discrimination of some species and so, confirmatory methods that extend the duration of the diagnosis are essential. The other genes whose sequences can be used as molecular targets for the detection and differentiation of *Babesia* species are genes encoding the Heat Shock Proteins, in particular, HSP70. However, the *hsp70* gene is largely conserved in its nucleotide sequence even between non-related organisms; therefore, this method, based on the amplification of the whole genome or its fragments, applies mainly to molecular phylogenetic analysis.

In conclusion, the selection of a genetic marker for PCR is very important for the sensitivity of this technique.

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