USEFULNESS OF TOUCH-DOWN PCR ASSAY FOR THE DIAGNOSIS OF ATYPICAL CASES OF BABESIA CANIS CANIS INFECTIONS IN DOGS

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Abstract

The aim of the present study was to evaluate usefulness of PCR assay for the diagnosis of atypical cases of the Babesia canis infections. Primers designed based on the 18 S RNA gene sequence of B. canis. The location of the primers was targeted to evolutionary conserved region. Blood samples from dogs suspected for babesiosis were analysed microscopically and then by PCR. The PCR assay showed higher efficacy and specificity of the parasite detection. It is concluded that the sensitivity of the touch-down PCR makes it particularly appropriate for diagnostics of atypical and chronic cases of babesiosis. The sequencing analysis of the studied isolates revealed that canine babesiosis in Warsaw area is caused by Babesia canis canis.

Key words: dog, Babesia, PCR.

Canine babesiosis has recently been recognized as an emerging infectious disease of dogs in Poland (11). Babesia canis and Babesia gibsoni are the major species which may cause babesiosis in dogs. Parasites from these two species can be distinguished in Giemsa stained blood smears as they differ in size. Babesia gibsoni is twice smaller (1.9 µm x 1.2 µm) than Babesia canis that is classified as a large piroplasm (2.5 µm x 5.0 µm). Babesia canis is found worldwide, being the most widespread and also pathogenic Babesia species in dogs. A typical intraerythrocytic piroplasma is pear-shaped and often occurs in pairs (13). In acute infection an erythrocyte may contain parasites in number from 4 to 16. In chronic infection amoeboid and ring forms can be observed.

Developments in genetic technology have contributed to the delineation between species and subspecies of Babesia organisms. Three subspecies of B. canis have been identified using RFLP (restriction fragment length polymorphism) analysis of PCR (polymerase chain reaction) when DNA coding for small subunit ribosomal RNA (ssrRNA) was amplified. These subspecies have been named Babesia canis canis, B. canis vogeli, and B. canis rossi (8). B. canis canis may be transmitted by tick Dermacentor reticulatus, B. canis vogeli is transmitted by Rhipicephalus sanguineus and B. canis rossi is transmitted by Haemophysalis leachi (12, 18, 22).

In recognition of the disease a proper diagnosis is very essential because in per-acute form of the disease it develops in 1-2 d. Young dogs and puppies are highly susceptible, with death occurring in the absence of treatment, and transplacental transmission has been suggested since high parasitaemia has been observed in neonates (5, 9, 15). Moreover, the clinical manifestations of infections caused by the three subspecies were found to differ significantly. B. canis rossi is highly virulent and causes either a haemolytic disease (possibly immune-mediated) or an acute and overwhelming inflammatory response (17) infection by B. canis canis results in transient parasitaemia, usually below 1% and clinical disease associated with congestion of internal organs (19), while infection by B. canis vogeli leads to a relatively mild disease, often without evident clinical signs. Therefore, on the basis of clinical symptoms it is difficult to put a proper diagnosis (2) and confirmation by a sensitive diagnostic test is required.

Babesiosis has been classically diagnosed by demonstrating intraerythrocytic trophozoites on Giemsa-Romanowsky stained blood smears. B. canis generally appears as a paired, piriform figure measuring 5 x 2-3 micrometers (Fig. 1). This is an insensitive procedure, particularly for animals in the carrier state. Babesia canis infections can also be determined using serological methods such as the indirect fluorescent antibody test (IFAT) (22, 23) or ELISA (12).
Recently, the development of PCR-based detection assays for some *Babesia* species have been described (1, 2, 10, 14, 24). The studies have shown these assays to be more sensitive than, and equally specific for the detection of acute cases as smear evaluation.

The aim of the present study was to develop the diagnostic test based on PCR assay allowing a proper diagnosis in cases when blood smear analysis is negative or dubious as well as to establish which of *Babesia canis* subspecies occurs in Central Poland.

**Material and Methods**

**Material.** Blood samples were collected from dogs of Warsaw area, showing clinical signs of babesiosis: depression, lethargy, fever (40-41°C), anaemia, accelerated respiration and tachycardia, vomiting, haematuria, and icterus. The dogs were of various breeds and of different age (from less than 6 months to more than three years).

**Microscopical examination.** Thin blood smears of peripheral blood samples were fixed for 3 min with methanol then stained with Giemsa solution for 25 min, washed and observed under microscope. Two hundred oil immersion fields were examined.

**DNA preparation.** Genomic DNA were extracted from the blood of individual dogs using blood DNA Prep Plus (A&A BIOTECHNOLOGY). The DNA samples were stored at -20 °C.

**Polymerase chain reaction (PCR).** PCR was performed using oligonucleotide primers (BeW-A: 5’-CATCTAAGGAAGGCAGG; BeW-B: 5’-TATAATGGAAACGTCCCTGGC) complementary to the region of 18 S ribosomal subunit DNA of *Babesia canis* submitted to the GenBank under accession No. L19079.

The reactions were carried out in 25 µl of mixture containing about 0.5 µg of template DNA, 1.25 U Taq polymerase (Madena Phurma Terpol Group), 1x buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl, 15 mM MgCl2), 200 µM of dNTPs, 25 pmol of each primer. Negative control reactions were carried out without DNA.

To obtain better specificity of the amplicons, touch-down PCR were performed. Reaction consisted of an initial 2 min denaturation step at 94°C, annealing at 60°C for 30 s, extension at 72°C for 30 s. In the next 2 cycles temperature of annealing was lowered about 2 degrees; for the next 15 cycles annealing was carried out at 54°C and in next 15 cycles at 52°C (denaturation at 94°C for 1 min; extension at 72°C for 30 s). Final extension was done at 72°C for 10 min.

Amplification products were subjected to electrophoresis and visualized on ethidium bromide-stained 1% agarose gel (Sigma-Aldrich).

**DNA cloning and sequencing.** For facilitation of subsequent cloning, *Eco*RI and *Pst*I restriction enzymes sides were incorporated into the 5’ ends of the BCW-1 and BWC-2 primers. Amplification was performed with three-step cycling program, as follows: 1 min of

denaturation at 94°C, 1 min of annealing at 51°C, 1 min of extension at 72°C. PCR 0.5 kb products were isolated from the agarose gel using Gel-Out kit (A&A BIOTECHNOLOGY), purified using DNA Clean-Up kit (A&A BIOTECHNOLOGY), digested with *Eco*RI and *Pst*I enzymes and ligated into plasmid vector pBluescript II SK(-) (Stratagene) and used to transform XL1-Blue *Escherichia coli* cells by electroporation. Transformants were checked by the digestion of plasmid DNA with *Eco*RI i *Pst*I enzymes.

Sequencing reactions were analysed on the ABI 3100 automatic DNA sequencer (Applied Biosystems).

**Results**

The majority of samples showed typical trophozoites of *Babesia canis* (Fig. 1), but approximately 1.5% of blood smears from dogs showing symptoms of babesiosis contained abnormal trophozoites (neither piriform nor ringform). These samples were subjected to DNA analysis by touch-down PCR method.

![Fig. 1. Giemsa stained blood smear from *B. canis canis* infected dog showing a typical trophozoite.](image-url)

To evaluate a usefulness of the touch-down PCR assay for definitive diagnosis of *B. canis* infections, we analysed 57 samples and among them 6 containing abnormal trophozoites. Following the PCR amplicons of 509 bp appeared in 4 samples with abnormal trophozoites (Fig. 2). Out of remaining 51 samples tested, in 9 PCR amplicons showing the presence of *B. canis* DNA were obtained while simultaneous microscopical analysis did not reveal any trophozoites. In five cases there was no PCR product although microscopic examination was positive. In such cases of incompatibility of these two diagnostic methods, PCR reaction was repeated and confirmation of earlier obtained results was achieved.
To determine the sensitivity of the PCR test, DNA extracted from the blood of infected dog was diluted, then used as the template for the test. On the basis of the dilution experiments it was found that our PCR test can detect parasitaemia on the level of 0.00015%.

The PCR amplicons were digested with EcoRI and PstI, cloned into pBluescript plasmid and sequenced. The three sequenced amplicons of ssrDNA possessed 509, 509 and 508 bp and in the BLAST analysis showed 100% similarity score with complete ssrRNA sequence of Babesia canis canis from France (accession No. AY072926), and 99% with sequence from Great Britain (AY272047).

The full Warsaw sequence of B. canis canis ssrDNA fragment was then submitted to GenBank (accession No. AY272047).

Discussion

Classical diagnosis of canine babesiosis relies mainly on microscopic identification of piroplasms in erythrocytes. However, in animals with chronic disease the number of parasites is too low to be detected by microscopic observation of Giemsa-stained thin blood smears (3). Effectiveness of this method is also limited by 10 day period when parasites disappear from peripheral blood (4). During this period DNA of parasites can be detected by PCR (10). The advantage of microscopic method is that it does not demand any specialist equipment but, on the other hand, it needs a very well trained and skilled microscopist.

Divergence among results observed in the present study may be an example of such lower sensitivity of microscopic method, which is able to detect the parasite in blood with parasitaemia of 0.001% (15). On the other hand, false positive results of microscopic method can be caused by artifacts (e.g. dye deposits in erythrocytes).

Touch-down PCR used in this study revealed higher specificity than a conventional PCR, six cycles were carried out conditions of higher temperature which gives higher specificity, and the next cycles under standard condition. The sensitivity of the test, determined by serial dilutions of DNA samples extracted from blood of dog with parasitaemia, was 0.15%. Parasitaemia detectable by our modification of the PCR was estimated as 0.00015%.

In 2001 Japanese scientists applied nested PCR for the detection of Babesia gibsoni – the most frequent etiological agent of canine babesiosis in Japan. This diagnostic test was designed to amplify a fragment of small ribosomal subunit gene, as in our study. The nested PCR with inner primers was carried out only when in the first reaction there was no PCR product because of small amount of DNA sample. The sensitivity of the test was established as 0.000118% performing serial dilutions of infected dog blood samples (1). Recently, it has been developed a seminested PCR test which was able to detect and discriminate B. gibsoni (Asian genotype), B. canis subsp. vogelli, B. canis subsp. canis, and B. canis subsp. rossi DNA in blood samples from infected dogs (6).

Sequencing results obtained in the present study suggest that in Central Poland only Babesia canis canis is present. Molecular analysis of full length ssr RNA from Italian dog which acquired infection during its stay in Poland confirmed this result (7).

Amplicons of two out of three sequenced samples had a length of 509 bp and the third one 508 bp. A BLAST search against GenBank revealed the highest similarity score (100%) with a partial, 365 bp long sequence from B. canis canis from France (accession No. AY072926) and Great Britain (99%, accession No. AY272047). Two out of three sequences analysed in the present study were identical in length and one was shorter. There were also two nucleotide substitutions. The observed differences may represent sequencing errors or minor strain variation. Similar variations in sequences of B. canis canis were reported by Zahler et al. (25).

The presence of B. canis canis in infected dogs from Poland is in agreement with the known distribution of the vectors of the subspecies (21). However, because of the expanding international mobility of pet dogs, and the presence of competent tick vectors this situation may change in the future. An increased contact of dogs with the wild environment may expose them to infection with new Babesia species (18) and a possible role of dogs as reservoirs of zoonotic disease can not be excluded.

References

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