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Original article

Identification of new 18S rRNA strains of *Babesia canis* isolated from dogs with subclinical babesiosis

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Abstract

In this study, we used PCR to detect and characterize *B. canis* from naturally infected dogs in Poland with subclinical babesiosis by amplifying and sequencing a portion of the 18S ribosomal RNA (rRNA) gene. Venous blood samples were collected from ten dogs with subclinical babesiosis. A 559-bp fragment of the *B. canis* 18S rRNA gene was amplified by PCR. Sequencing of the PCR products led to the identification of a new variant of *Babesia canis*, differing from the previously detected protozoa genotypes (18S rRNA-A and 18S rRNA-B) with nucleotide substitutions in positions 150 and 151 of the tested gene fragment. The results indicate the emergence within the Polish territory of a new, previously unencountered *Babesia canis* genotype responsible for the development of subclinical babesiosis.

Key words: *Babesia canis*, 18S rRNA, PCR, dog, subclinical infection

Introduction

Canine babesiosis is a common and clinically significant tick-borne disease caused by hematozoan parasites of the genus *Babesia* (Adaszek and Winiarczyk 2008a). The classification of *Babesia* spp. places them in the order Piroplasmida within the phylum Apicomplexa. Two morphologically distinct forms of the erythrocytic stage in the canine host were recognized in earlier studies, leading to the naming of the larger form measuring approximately 3-5 μm as *B. canis*, and the smaller (1-3 μm) as *B. gibsoni*. On the basis of cross-immunity, serological testing, vector specificity and molecular phylogeny *Babesia canis* was reclassified into three sub-species: *B. canis canis, B. canis rossi*, and *B. canis vogeli*. All of these are now considered to be separate species (Zahler et al. 1998, Matijatko et al. 2012). So far, only *B. canis* has been found in dogs in Poland (Zygner et al. 2007, Adaszek et al. 2011). These parasites are also the most common etiologic factor of babesiosis in dogs in other parts of Europe (Cardoso et al. 2008, Solano-Gallego et al. 2008, Kubelová et al. 2011). Clinically, these pathogens cause remittent fever, progressive anemia,

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hemoglobinuria, marked splenomegaly and hepatomegaly in dogs and, in some cases, the death of the infected animals (Milczak et al. 2004, Adaszek et al. 2009).

Standard diagnostics for babesiosis in the areas of its enzootic occurrence are based on information from the owners (presence of ticks on the dog), clinical signs and microscopic assessment of blood smears of sick animals, stained according to the Giemza (Casapulla et al. 1998, Schuster 2002, Adaszek and Winiarczyk 2008a), Romanowski (Ewing 1963) or Diff-Quick (Suarez et al. 2001) methods.

Methods of molecular biology are being used with increasing frequency, especially for the polymerase chain reaction and sequencing of amplification products, in the diagnosis of babesiosis and the assessment of the epizootic situation, including the detection of subclinical infections with these protozoa (Macintire et al. 2002).

The previous genetic analysis of isolates of *Babesia canis* from the different areas of Poland helped to distinguish two genotypes within the species: 18S rRNA-A and 18S rRNA-B (Adaszek and Winiarczyk 2008a). A certain interrelation between the intensification of thrombocytopenia and the fact that the protozoa either belong to groups A or B was presented. The mean number of thrombocytes in dogs infected with protozoa from the 18S rRNA-B group was significantly lower than in dogs of the 18S rRNA-A group. A strong correlation was also observed between the low level of thrombocytes and an increase in internal body temperature, accelerated pulse rate and discoloration of the urine (Adaszek et al. 2009).

In this paper we present the identification of a new genotype of *Babesia canis* detected in dogs in Poland having subclinical babesiosis.

Materials and Methods

Animals used in the study

The study included ten dogs (6 males and 4 females) of different breeds (3 crossbreeds, 2 Labradors, 2 German Shepherds, 1 American Staffordshire Terrier, 1 Weimaraner, 1 Irish Setter), aged 3-8 years with subclinical babesiosis. The animals were referred to the Clinic of Infectious Diseases at the University of Life Sciences in Lublin with recurrent periods of weakness, occasionally accompanied by symptoms of abdominal pain. Hematologic tests of all the dogs revealed thrombocytopenia, which, in the case of three animals, was accompanied by anemia. Biochemical tests did not show impaired liver function or impaired renal function in any of the patients (AST, ALT and AP activity, as well as urea and creatinine concentration, remained within physiological limits). Abdominal radiographs of four dogs showed enlargement of the spleen. Microscopic examination of blood smears stained using the Giemsa method did not detect *Babesia* protozoa in erythrocytes or rickettsia *Anaplasma*/*Ehrlichia* in the leukocytes in any of the test subjects. Interviews with the owners of the animals showed that all the dogs had had contact with ticks in the past. The first symptoms of the disease occured 6 to 14 days after exposure of the animals to the ticks. Recurrent periods of weaknes were reported every 3-6 weeks. Due to the possibility of developing subclinical infections with *Babesia*/*Ehrlichia*/*Anaplasma,* all the patients were subject to molecular testing (PCR) in order to detect the genetic material of pathogens in their blood.

Molecular analysis for babesiosis

DNA for molecular analysis was extracted from EDTA-anticoagulated whole blood using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) or the Blood Mini DNA isolation kit (A&A Biotechnology, Gdańsk, Poland) according to the manufacturer's instructions. The amplification of *B. canis* DNA through PCR was performed using the forward primer BAB GF2 (5'-GTC TTG TAA TTG GAA TGA TGG-3') and the reverse primer BAB GR2 (5'-CCA AAG ACT TTG ATT TCT CTC-3'), which amplify a 559-bp region of the 18S rRNA gene of *B. canis* (Adaszek and Winiarczyk, 2008a).

In brief, each reaction mixture (50 µL) contained 100 µM of each dNTP, 1.6 mM of MgCl2, 0.25 µM of each primer, 2.5 U of *Taq* DNA polymerase, and 5 µL of DNA template. PCR amplification was performed using a programmable thermal cycler (Biometra, Goettingen, Germany) with the following program: an initial denaturation at 92°C for 2 minutes, 50 cycles of denaturation at 92°C for 60 seconds, annealing at 52°C for 60 seconds, and extension at 72°C for 90 seconds, followed by a final extension at 72°C for 5 minutes. Positive and negative controls were included in all amplifications. The PCR results were evaluated by agarose gel (1%) electrophoresis stained with ethidium bromide in parallel with a 100-bp DNA ladder (Gibco/BRL, Gaithersburg, MD, USA).

The PCR products were purified using QIAquick spin columns (Qiagen) and eluted in 50 µl of Tris 10 mM, pH 7.6. The DNA sequence was determined on both strands using the same primers employed for PCR at a DNA sequencing core facility (Research Institute, Polish Academy of Sciences, Warsaw,

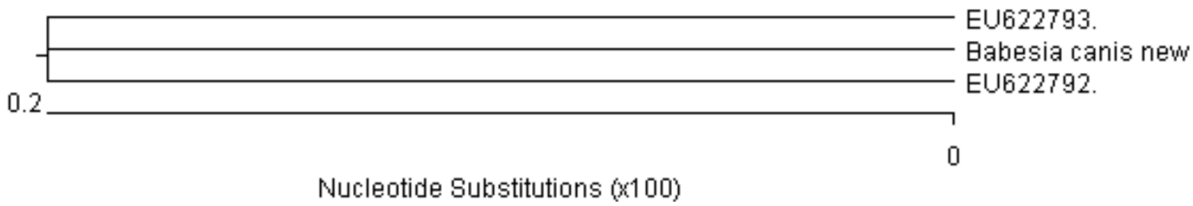

Fig. 1. Phylogenetic relationships between sequences of the *B. canis* isolates from our own study and EU622792 and EU622793 sequences deposited in GenBank.

Poland). The DNA sequences were assembled and edited using SeqMan (DNAstar, Lasergene, Madison, USA), and ClustalV alignments to the published *B. canis* 18S rRNA-A and 18S rRNA-B (GenBank accession numbers: EU622792 and EU622793).

Results

For all dogs the PCR results for *Ehrlichia*/*Anaplasma* were negative; however, for all ten animals the PCR study revealed the presence of the genetic material of *B. canis* of length 559 bp. All amplicons were sequenced in order to identify the molecular heterogeneity of the different isolates**.** The nucleotide sequences obtained were characterized by high mutual homology (99.1-100%) of the tested section of the 18S rRNA gene and a relatively high similarity (98.3-98.8%) to 18S RNA genotypes A and 18S rRNA-B *Babesia canis* (EU622792 and EU622793), which were previously detected and described in dogs suffering from babesiosis in Poland. A detailed analysis of the nucleotide sequence of the tested gene fragment revealed differences in positions 150 and 151 between the isolates from our own studies and sequences EU622792 and EU622793. In the investigated 18S rRNA gene sequence all isolates of the EU622792 group have guanine in position 150 and adenine in position 151, whereas the isolates of the EU622793 group have adenine in position 150 and guanine in position 151. All *Babesia* strains obtained from the ten dogs with subclinical babesiosis had thymine in positions 150 and 151 of the tested section of the 18S rRNA gene. A phylogenetic tree (Fig. 1), whose three clearly separated branches are formed by the *B. canis* EU622792 and EU622793 genotypes obtained in the present study constitute a graphical representation of the nucleotide differences.

Discussion

Among the *Babesia* species pathogenic for dogs, *B. canis* is considered to be the most pathogenic (Hauschild and Schein 1996). These parasites can also be isolated from individuals without the tell-tale signs of clinical babesiosis (Wlosniewski et al. 1997, Carret et al. 1999, Beck et al. 2009). Animals with an asymptomatic invasion may constitute a reservoir of protozoa and play an important role in the epidemiology of the disease (Ionita et al. 2012). It is assumed that the frequency of determination of subclinical infections in dogs is small. Beck et al. (2009) found it in only 29 out of 848 dogs (3.42%) not showing clinical symptoms of the disease. In Poland, the first monitoring of this type of invasion in dogs using the methods of molecular biology was conducted by Welc-Falęciak et al. (2009). Their results indicate that subclinical babesiosis can be a more significant problem than is commonly assumed. The authors demonstrated the presence of the DNA of *B. canis* in the blood of 20 out of the 79 (25.3%) apparently healthy individuals used in the study. These results, although obtained from a small group of animals, indicate a significant risk of this type of invasion developing in domestic dogs and the associated need to develop a sensitive diagnostic method able to detect subclinical infections.

The standard diagnosis of canine babesiosis is based on the microscopic evaluation of blood smears stained using the Giemsa, Romanowski, or Diff-Quick methods (Adaszek and Winiarczyk 2008b). The examination of blood smears, however, requires some experience. Unwashed dye components may sometimes be mistaken for protozoa cells. Furthermore, at an early stage of the disease while parasitemia is still low it can be difficult to demonstrate the presence of *Babesia* in the erythrocytes of infected dogs. A similar situation occurs with severe hemolysis, when it involves the simultaneous breakdown of many red blood cells and the release of parasites into the plasma in the course of subclinical infections (Adaszek and Winiarczyk 2008b, Taboada and Lobetti 2010). All this results in the fact that in cases of suspected babesiosis, in which microscopic evaluation of blood smears yields a questionable result, it is advisable to test this material using the PCR method. PCR is characterized by much higher sensitivity than the microscopic examination of blood smears (Bashiruddin et

al. 1999). It allows one to demonstrate the presence of *Babesia* genetic material in parasitemia at levels of only 0.0001% (Ano et al. 2001). Moreover, with its help, it is possible to differentiate those parasites which do not show an altered cellular morphology (Criado-Fornelio et al. 2003). The sensitivity of this technique depends on the amount of template DNA, and that amount depends, in turn, on the efficiency of the method of nucleic acid isolation (McPherson et al. 1991).

The results obtained confirm the usefulness of PCR in the diagnosis of subclinical canine babesiosis. So far, no comprehensive monitoring of this form of babesiosis has been conducted in Poland. All parasites detected in our own studies were classified as belonging to the as yet unnamed *Babesia canis* strain. These results may indicate the appearance of a new, so far unknown, *Babesia canis* genotype within the Polish territory. Until recently, the main cause of canine babesiosis in native dogs involved the 18S rRNA-A EU622792 strains (Adaszek et al. 2012), which is why the detection of a new genotype of protozoa constitutes a confirmation of the changes that occur in the genome of the parasites, which may, in turn, translate into a changed clinical course of the disease (Adaszek and Winiarczyk 2008a, Adaszek et al. 2009, Adaszek and Winiarczyk 2010). In order to determine whether this variant of *Babesia* is responsible for the development of subclinical babesiosis, it is advisable to conduct further work, including the molecular monitoring of *Babesia canis*.

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