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Occurrence of different strains of *Babesia canis* in dogs in eastern Poland

Paweł Łyp, Michał Bartnicki, Marta Staniec, Stanisław Winiarczyk, Łukasz Adaszek

Department of Epizootiology and Infectious Diseases, Faculty of Veterinary Medicine, University of Life Sciences Lublin, 20-612, Lublin, Poland
pawel.lyp86@gmail.com

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Abstract

Introduction: The aim of this study was to carry out a genetic analysis of *Babesia canis* isolates detected in dogs in eastern Poland and to study the correlation of the protozoa variant with a specific geographical region. **Material and Methods:** PCR was used to identify strains of *B. canis* from naturally infected animals (240 dogs from four provinces: Mazowieckie, Lublin, Podlasie, and Podkarpacie) by amplifying and sequencing a fragment of the 18S rRNA gene. **Results:** Sequencing the PCR products led to the identification of four variants of *B. canis*. Two previously described protozoa variants (18S rRNA-A and 18S rRNA-B) were observed in all provinces. Additionally, in the Mazowieckie and Lublin provinces a *B. canis* variant which contributed to the development of acute or atypical babesiosis was observed. The fourth variant of *B. canis* was detected only in dogs from the Lublin province, and the course of the disease was subclinical in all dogs infected with this variant. **Conclusion:** These results indicate the appearance of a new fourth *B. canis* genotype in Poland and confirm that it is still necessary to study the relationships between the genetic structure of protozoa, geographical distribution of the parasites, and clinical course of the disease.

Keywords: dog, *Babesia canis*, 18S rRNA, PCR, Poland.

Introduction

Canine babesiosis is a disease caused by parasites of the genus *Babesia* (1). Numerous species of *Babesia* exist worldwide. Parasite blood smears have recognised them as forming two groups: the large form (3–5 μm) – *B. canis***,** and the small form (1–3 μm) – *B. gibsoni***.** Based on its geographic distribution, hosts, modes of transmission, pathogenicity, and the results of molecular studies, *B. canis* has been reclassified into four species: *B. canis***,** *B. rossi, B. vogeli,* and the unnamed *Babesia* sp. detected in North America (7, 15, 18). All of these pathogens cause fever, pigmenturia, and haemolytic anaemia. In particular cases the outcome of the disease may be fatal (4, 13). Moreover, significant deviations from the standard results of serum biochemical tests may be observed in infected dogs (20).

Blood smear examination is a useful diagnostic tool for detection of clinical babesiosis in dogs. Microscopic evaluation continues to be the easiest and most accessible diagnostic test for most veterinary practitioners. However, the sensitivity of this method is lower than that of molecular diagnosis. Although light microscopy is highly specific and can be used to diagnose the majority of dogs infected by the large forms of *Babesia* (*e.g. B. canis*), it is less effective at detecting *B. gibsoni* infections. The small piroplasms are hard to observe using light microscopy, which has relatively poor to moderate sensitivity, and expertise is required (16).

PCR and the sequencing of amplification products are being used with increasing frequency in the diagnosis of babesiosis and the assessment of the epizootic situation, including the detection of subclinical infections with *B. canis* protozoa (14). The genetic analysis of Polish isolates of *B. canis* allowed three groups of parasites to be identified: 18S rRNA-A, 18S rRNA-B (1), and the new and yet-unnamed 18S rRNA strain responsible for subclinical babesiosis (13).

The aim of this study was to perform a genetic analysis of *B. canis* isolates detected in dogs in eastern Poland and to possibly identify whether the occurrence of each protozoa strain could be correlated with a specific geographical region.

Material and Methods

Animals. The study was conducted in four eastern Polish provinces (Lublin, Mazowieckie, Podlasie, and Podkarpacie) from March 2015 to March 2016. It included 240 dogs (60 animals from each province, 182 males and 58 females, with ages ranging from 6 months to 11 years) with babesiosis confirmed by blood smear analysis and/or the results of PCR. In 198 animals, clinical examination revealed symptoms of acute babesiosis (fever, weakness, pallor of mucous membranes, and jaundice). Other dogs diagnosed showed atypical signs of the disease (anaemia and weakness), or their clinical examination did not reveal any abnormalities (subclinical infection). The dogs with subclinical infection were classified based on the presence of the ticks on their bodies and the results of haematological examination. All animals had thrombocytopenia (PLT = 12–88 × 10^9/L) and mild anaemia (4.5–5.3 × 10^{12}/L).

Characteristics of provinces. Lublin province is situated mainly between the Vistula and Bug Rivers, and is divided into three geographically diverse areas: the hilly Lublin Upland with ravines; the forested Lublin Lowlands, and Leczna/Wlodawa with its large lakes. Mazowieckie is a low-lying region 57–325 m above sea level and is divided by the three main rivers: the Vistula, Bug, and Narew. Podlasie is an area lower than 299 m above sea level, topographically marked by postglacial lakes, marshland, and peat bogs. Podkarpacie in the south-eastern corner of Poland has a diverse natural environment.

The climate of these provinces is mostly continental, with average temperatures of 7 to 8ºC, average annual precipitation from 500 to 1200 mm, and a growing season ranging from 180 to 220 days. The mainly coniferous forests cover 21.7%–39.5% of the regions, arable soil by area is 37.2%–55.9%, and meadows spread over 7.7%–20.0%.

Collection of blood samples and their analysis. The study was performed at the Clinic of Infectious Diseases, Faculty of Veterinary Medicine, University of Life Sciences in Lublin. Whole blood (EDTA) was taken from all animals for microscopic examination as well as molecular (PCR) tests. One drop of blood from each sample was used to prepare blood smears stained by the May-Grünwald-Giemsa method. The remaining blood was frozen at −20ºC for further analysis – DNA extraction for PCR using a DNA Blood kit (A&A biotechnology, Poland).

Molecular analysis. Amplification of the fragment of the 18S rRNA of *B. canis* gene with a size of 559 bp was performed using the pair of primers: BAB GF2 (5'-GTC TTG TAA TTG GAA TGA TGG-3') and BAB GR2 (5'-CCA AAG ACT TTG ATT TCT CTC-3') (1).

PCR was carried out in 50 μL of total reaction volume containing: 100 μM of each dNTP, 1.6 mM of $MgCl_2$, 0.25 μM of each primer, 2.5 U of *Taq* DNA polymerase, and 5 μL of DNA template. Amplification of parasite DNA was performed in a Biometra thermocycler (Germany). Cycling conditions for *B. canis* were: initial denaturation at 92ºC for 2 min, followed by 50 cycles of denaturation at 92ºC for 60 s, annealing at 52ºC for 60 s, extension at 72ºC for 90 s, and a final extension at 72°C for 5 min. Positive and negative controls were included in all amplifications. The PCR products were separated by electrophoresis on 1% agarose gel and visualised using ethidium bromide (1 μg/mL).

The amplicons were purified on QIAquick spin columns (Qiagen, USA) and eluted in 50 μL of 10 mM Tris, pH 7.6. The DNA sequence was determined on both strands using the same primers as for PCR at a DNA sequencing core facility (Research Institute, Polish Academy of Sciences). The DNA sequences were assembled and edited using SeqMan (DNAstar, Lasergene, USA), and ClustalV alignments.

Results

Confirmation of babesiosis. Analysis of blood smears confirmed babesiosis in 198 animals (82.5%). In each case merozoites or trophozoites of *Babesia* were observed within red blood cells. The positive results of the microscopy test were confirmed by positive results of PCR in each case. All dogs demonstrated symptoms of acute babesiosis, including: fever, weakness, pallor of mucous membranes, jaundice, or haemoglobinuria.

Microscopic blood examination in the remaining 42 dogs did not demonstrate the presence of protozoa in the erythrocytes. The infection in the animals was confirmed only by PCR results. The course of babesiosis in 31 animals was non-typical, with the dominating symptoms of anaemia and severe weakness recurring every few days. The pet owners reported that the symptoms prevailed for about five to seven weeks. In the remaining 11 dogs no symptoms of babesiosis were observed. All dogs in this group had a history of contact with ticks.

Results of molecular analysis in connection with the clinical course of the disease. A fragment of *B. canis* DNA with a size of 559 bp was detected in all animals. The analysis of the 18S RNA gene fragment sequence helped to identify four polymorphic groups of protozoa. Isolates of *B. canis* with a common sequence were classified into the first group to a total of 107, demonstrating 100% homology with the previously described *B. canis* 18S rRNA sequence EU622792 available in the NCBI GenBank (Fig. 1).

Fig. 1. Nucleotide sequence of 18S rRNA gene fragment from own isolates classified in the first group. Position of mutations in polymorphic groups 2, 3, and 4 are marked

3'-GTCTTGTAATTGGAATGATGGTGACCCAAACCCTCACCAGAGTAGCAATTGGAGGGCAAGTCTGGTGCCAGCAGCC
GCGGTAATTCCAGCTCCAATAGCGTATATTAAACTTGTTGCAGTTAAAAAGCTCGTAGTTGTATTTTTGCGTTAGCGGTT
TGACCATTTGGTTGGTTATTTCGTTTTCGCTTTTGGGAATTTCCCTTTTTACTTTGAGAAAATTAGAGTGTTTCAAGCAGA
CTTTTGTCTTGAATACTTCAGCATGGAATAATAGAGTAGGACTTTGGTTCTATTTTGTTGGTTATTGAACCTTAGTAATG
GTTAATAGGAACGGTTGGGGGCATTCGTATTTAACTGTCAGAGGTGAAATTCTTAGATTTGTTAAAGACGAACTACTGC
GAAAGCATTTGCCAAGGACGTTTCCATTAATCAAGAACGAAAGTTAGGGGATCGAAGACGATCAGATACCGTCGTAGT
CCTAACCATAAACTATGCCGACTAGTGATTGGAGGTCGTCGTTTTTTGACCCCTTCAGGAACTTGAGAGAAATCAAAGT
CTTTGG-5'

Isolates of protozoa demonstrating 100% similarity with the *B. canis* 18S RNA sequence EU622793 available in NCBI GenBank were classified into the second group in 84 cases. In the investigated 18S rRNA gene sequences 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. A microscopic examination revealed the presence of the protozoa in the blood of all dogs infected with the two strains mentioned, and the animals demonstrated symptoms of acute infection.

Group three covered 38 isolates characterised by 100% mutual homology. All *Babesia* isolates obtained from this group had thymine in positions 150 and 151. A microscopic test of blood samples from 31 dogs in this group did not reveal the presence of protozoa in the erythrocytes, and the course of the disease was non-typical. Dogs infected with the other seven strains of this group demonstrated symptoms of acute babesiosis and the haematological examination revealed the presence of merozoites in the erythrocytes.

Table 1. The differences in the nucleotide substitutions in the *B. canis* 18S rRNA gene fragment in four polymorphic groups detected in the study

Polymorphic group	Number. of isolates	Mutation	Position
No. 1	107	-	-
No. 2	84	GA→AG	150, 151
No. 3	38	GA→TT	150, 151
No. 4	11	A→C	221
		G→C	222
		A→C	223
		G→C	224
		T→C	225
		G→A	236
		G→T	258
		T→G	299
		A→G	357
		G→C	387
		G→T	473

Group four included 11 isolates of *B. canis*. The haematological examination did not reveal the presence of *Babesia* merozoites in any of the dogs infected with these parasites. Typical clinical symptoms of babesiosis were not observed in the animals either. The differences in the nucleotide substitutions in the representatives of each group are presented in Table 1.

Results of the epidemiological study. Table 2 presents the occurrence of each protozoa group in the dogs from the provinces examined.

Table 2. The occurrence of each protozoa group in the dogs by province

Province	Group 1	Group 2	Group 3	Group 4	Total
Lublin	16	18	15	11	60
Mazowieckie	17	20	23	-	60
Podkarpacie	39	21	-	-	60
Podlasie	35	25	-	-	60
Total	107	84	38	11	240

In the Podlasie and Podkarpacie provinces only cases of acute babesiosis were reported, and protozoa from groups 1 and 2 (EU622792 and EU622793) were isolated only from the sick dogs.

The Lublin and Mazowieckie provinces showed the presence both of strains developing acute babesiosis and strains developing an atypical disease course. The acute strains comprised the first and second groups (EU622792 and EU622793) and in Lublin province, half of the third group. The atypical strains accounted for the other half of the third group in Lublin's case but for the whole third group in regard to Mazowieckie.

The presence of representatives of three groups responsible for the development of acute babesiosis (representatives of groups 1 and 2 and seven isolates of group 3) and the atypical form of the disease (the isolates of group 3), as well as subclinical babesiosis (all isolates of group 4) were observed only in the Lublin province (Table 3). The full spectrum of disease courses was present only in the Lublin province. The strains giving rise to acute babesiosis (the isolates of groups 1 and 2 and half of group 3), strains causing atypical course (half of group 3), and strains inflicting only subclinical babesiosis (group 4) were all present here (Table 3).

Table 3. The association between the clinical course of the disease in dogs, and the strain of protozoa responsible for the development of babesiosis

		Number of isolates			
Group	Course of the disease	Lublin	Mazowieckie	Podlasie	Podkarpacie
1	Acute	16	17	35	39
2	Acute	18	20	25	21
3	Acute/atypical	7/8	0/23	0/0	0/0
4	Subclinical	11	0	0	0
	Total	60	60	60	60

Discussion

As demonstrated by the results of many previous studies, canine babesiosis is a disease endemic to Poland (3, 5, 20, 21). This situation is a result of *Dermacentor reticulatus* ticks becoming more widespread, because these ticks transmit the parasites (5, 21). A few years ago the disease was diagnosed only in the eastern part of Poland, and the results of this study show that these regions are indeed areas of endemic babesiosis (5, 9).

PCR is very useful in diagnosing babesiosis. Firstly, PCR detection is more sensitive than a direct blood smear examination. Secondly, the detection of DNA of a specific pathogen can be considered as evidence of an active – and therefore ongoing – infection. In addition to this, unlike direct detection by light microscopy or serological examinations, PCR allows for a more reliable identification of the causative species infecting the dog. PCR is a useful screening strategy considering that many dogs remain chronically infected with piroplasms. Their chronically infected status predisposes these dogs to relapse or to fail to improve from a chronic clinical state. Under these circumstances, PCR can be used to establish whether the infection remains or not (16).

Until now, only one *Babesia* species – *B. canis* – had been diagnosed in dogs in Poland (10). Previously, when the 18S rRNA gene was analysed, two genotypes of these protozoa responsible for the development of the acute form of the disease were identified; they are referred to as 18S RNA-A and 18S RNA-B (2, 3, 8). However, recent studies by Łyp *et al.* (13) revealed the presence of another genotype of the protozoa in Poland, which caused subclinical babesiosis in 10 dogs. Patients with this type of infection may serve as a source of *Babesia* parasite that is important from an epidemiological point of view (11).

As shown by the results of other studies, the frequency of asymptomatic infections in dogs is low. Beck *et al.* (6) found subclinical babesiosis in only 29 out of 848 (3.42%) dogs not showing clinical symptoms of the disease. The observation of Welc-Falęciak *et al*. (17) conducted in Poland showed that the subclinical form of the disease may be a more significant problem than is generally assumed. Using PCR, they detected the genetic material of *B. canis* in the blood of 20 out of 79 (25.3%) apparently healthy dogs. These results, although obtained from a not very large group of patients, indicate a significant risk of this type of infection in dogs and the need to develop sensitive diagnostic tests able to confirm subclinical infections.

The results of the present study partly confirm the previous observations by Łyp *et al.* (13) that the strain of protozoa discovered in 2015 is actually responsible mainly for the development of the atypical form of piroplasmosis; however, in seven dogs the infection resulting from this strain induced the development of a severe form of the disease.

Furthermore, our own studies also revealed the existence of another protozoa genotype responsible for the development of disease with a subclinical course, whose occurrence has so far been limited to the Lublin region. This indicates a continuous genetic evolution of the protozoa and the formation of new strains, where changes in the genome are related to changes in strain virulence (8).

Analysing the sequence of *B. canis* isolates obtained during our study with regard to the geographical area where they were collected, a pattern can be discerned. Although in the whole area of eastern Poland, similar to the whole of Europe (2, 8), the presence of EU622792 and EU622793 is observed and the genotypes are responsible for the development of acute babesiosis in dogs. The protozoa isolates responsible for the development of the atypical and subclinical forms of the disease have only been discovered in the Lublin and Mazowieckie provinces, where the number of cases of the disease seems to be the highest in Poland (5, 19).

These results indicate the appearance of new *B. canis* genotypes in Poland. It is still necessary to study the relationship between the genetic structure of protozoa, their geographical distribution, and the course of the disease in dogs (3). This analysis could provide the basis for developing a map of the progress of the disease among dogs in Poland and will also help to identify some factors facilitating the development of the infection.

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