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The first case of *Babesia gibsoni* infection in a dog in Poland

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ABSTRACT: Canine babesiosis is a tickborne, protozoal, haemoparasitic disease that can cause varying degrees of haemolytic anaemia, splenomegaly, thrombocytopenia and fever. Babesia organisms are frequently classified as either large or small. All small Babesia infections were previously attributed to *B. gibsoni*, but molecular analysis and DNA sequencing have revealed that there are at least three small piroplasms which infect dogs. Correctly identifying the infectious agent is important for treatment planning and prognosis. In this report, the first case of *Babesia gibsoni* infection in a Polish dog is presented.

Keywords: tickborne; molecular analysis; DNA sequencing; vector-borne diseases

Canine babesiosis is a disease caused by infection with parasites of the genus *Babesia* (Vial and Gorenflot 2006). Numerous species of *Babesia* exist worldwide. Two groups of these parasites have been identified, leading to classification into a larger (3–5 μm, *B. canis)* and smaller form (1–3 μm, *B. gibsoni).*

So far, only infections with the large *Babesia* species have been described in Polish dogs (Lyp et al. 2015).

Blood smear examination is a useful tool for diagnostics of clinical babesiosis in dogs, but microscopy evaluation continues to be the easiest and most accessible diagnostic test for most veterinarians. However, the effectiveness of this method in assisting the veterinarian to make a positive diagnosis is lower than that of molecular diagnosis and is rather dependent on the species infecting the dog. The two forms of *Babesia*, large and small, may be distinguished using a blood smear. Although light microscopy is highly specific and can be used to diagnose the majority of sick dogs infected by the large forms of *Babesia* (e.g., *B. canis*), it is less commonly used to detect *B. gibsoni* infections. The small piroplasms are hard to observe by light microscopy, which has relatively poor-to-moderate sensitivity, and a higher level of expertise is needed (Solano-Gallego et al. 2016).

Polymerase chain reaction-based assays and the sequencing of amplification products are being increasingly used in the diagnosis of babesiosis and for the assessment of the epizootic situation, including the detection of subclinical infections (Lyp et al. 2016).

The aim of this paper was to present a description of the first case of babesiosis caused by *Babesia gibsoni* detected in a dog in Poland.

MATERIAL AND METHODS

The subject of the study was a 4-year old American Staffordshire terrier presented to the veterinary clinic with symptoms of severe weakness, apathy and haematuria. The owners did not report the presence of ticks, nor had they recently travelled abroad with the animal. The dog was fed in the standard way and regularly subjected to prophy-

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laxis against ectoparasites, internal parasites and vaccinations against primary infectious diseases.

An ultrasonographic examination of the abdomen and pelvis was performed in this dog and its blood was collected for haematological and biochemical studies in order to run quick serological tests for anaplasmosis, ehrlichiosis, borreliosis and dirofilariasis and molecular tests for anaplasmosis/ehrlichiosis and babesiosis.

Haematological tests. Blood for the haematological tests was transferred to test tubes with EDTA and tested in an Exigo analyser (Boule Medical AB, Sweden).

Blood smear tests. Blood smears were conducted on degreased microscopic glass; smears were stained according to the Giemsa method and viewed under an Olympus CH 20 microscope when dry.

Biochemical tests. Blood for biochemical tests was transferred to test tubes with a coagulation accelerant, centrifuged and the obtained serum was tested using a Mindray BS-130 analyser (Mindray Co., Ltd).

Serological tests for anaplasmosis, ehrlichiosis, borreliosis and dirofilariasis. These tests were performed according to the procedure outlined by the manufacturer (Idexx Laboratory, France).

DNA isolation. DNA for analysis was extracted from 100 ml of fresh anticoagulated blood. DNA isolation was carried out with a DNA blood mini kit (A&A Biotechnology Gdynia, Poland).

PCR. The PCR blood test for *Babesia/Theileria* spp. was carried out using a pair of primers – GR2 and GF2 – which amplify a 559-bp fragment of the conserved 18S rRNA gene (Adaszek and Winiarczyk 2008). The EHR 521 and EHR 747 primers for *Ehrlicha*/*Anaplasma* spp. amplified a 247-bp fragment of the 16S rRNA gene of rickettsia (Adaszek et al. 2009).

Electrophoresis. PCR results were evaluated using agarose gel electrophoresis with ethidium bromide staining; a 100-bp DNA ladder was run in parallel (Gibco//BRL, Gaithersburg, USA).

Sequencing. The PCR products were purified using QIAquick spin columns (Qiagen) and eluted in 50 µl of 10 mM Tris buffer, 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 of the Polish Academy of Sciences in Warsaw, Poland). DNA sequences were assembled and edited using SeqMan (DNAstar, Lasergene, Madison, USA), and ClustalV alignments were made with the published 18S rRNA gene sequences in the NCIB GeneBank for *B. gisoni* LC012808.1, *B. canis* EU622792 and EU622793.

RESULTS

The low number of erythrocytes (3.76×10^{12}/l), low content of haemoglobin in erythrocytes (8.24 g/l), decreased haematocrit (35.4%), thrombocytopaenia (53.0×10^{12}/l) and the increase in the bilirubin levels (2.1 g/dl) show that the tested animal suffered from haemolytic anaemia. The white blood cell count (14.6×10^{9}/l) was within the standard physiological ranges. High levels of ALT (1653 mkat/l) and AST (1152 mkat/l) activity in the blood serum indicated impaired liver function.

Microscopy evaluation of the blood smears stained using the Diff-Quik method demonstrated the presence of intracellular inclusions that were identified as *Babesia* protozoa. However, the presence of *Anaplasma/Ehrlichia* inclusions in the leukocytes was not confirmed. Quick diagnostic tests for borreliosis, ehrlichiosis, anaplasmosis and dirofilariasis were negative. The ultrasonographic examination revealed a hypoechogenic liver with a thickened gallbladder, filled with bile containing sludge. Using the PCR method, the DNA of *Babesia* spp. was detected in the blood of the sick dog. The PCR results for *Ehrlichia*/*Anaplasma* were negative. The sequences of the 559-bp-long *Babesia* spp. products obtained in the PCR and analysed in DNASTAR Lasergene showed a high similarity (99.8%) to the *B. gibsoni* LC012808.1 sequence and 88.6% homology with *B. canis* EU622792 and EU622793 sequences.

The dog was administered imidocarb (5 mg/kg); however, pharmacotherapy was not effective. The protozoa continued to persist in the erythrocytes, and the condition of the dog deteriorated. The results of the laboratory tests worsened until they reached extreme values (WBC = 59×10^{9}/l, RBC 1.3×10^{12}/l, Hb 2.4 g/dl). Interestingly, the platelet count, although still below normal, increased significantly compared to the first study and remained within the range of $140–170 \times 10^{12}$/l. Due to the ineffectiveness of the imidocarb, the dog was given the antimalarial drug Malarone (proguanil + atavaquone, ½ tablet twice a day) in addition to

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azithromycin (250 mg once daily). A coordinated treatment regimen improved the animal's health. Unfortunately, after four to five weeks the symptoms returned and laboratory tests confirmed the invasion of *B. gibsoni*. When the next treatment with Malarone was attempted, the dog showed symptoms of urticaria, paw oedema and itching of the eyes; therefore, further treatment with the aforementioned drugs was discontinued and the following combination of drugs was introduced: diminazen 5 mg/kg (once daily), metronidazole 25 mg/kg every 8–12 hours for three weeks, clindamycin 12.5 mg/kg twice daily for three weeks. The applied therapy led to a significant improvement in the health status of the dog. Complete recovery of the infected animal was observed after three weeks of therapy. The results of a control PCR test performed three weeks after the end of the treatment were negative for the presence of *Babesia gibsoni* genetic material in the blood of the dog. A haematological test carried out one month after the initiation of the treatment did not reveal any abnormalities.

DISCUSSION

In this report, we have described the first laboratory-confirmed case of *B. gibsoni* infection in a dog in Poland. It may seem strange that the disease developed in an animal that never left the country. It is important to note that *B. gibsoni* is transmitted by *R. sanguineus* and *Haemaphysalis longicornis* ticks (Hatta et al. 2013; Iwakami et al. 2014); the former is found in Poland only occasionally, while the latter is especially established in Asia. The fact that the probability of the dog coming into contact with the vector is minimal also makes this clinical case more interesting. From a purely theoretical point of view, it is impossible to exclude the possibility of contact between the dog and infected *R. sanguineus* and *Haemaphysalis* arachnids, which could have arrived in Poland on ships, for example. The patient came from the coast, so contact with a vector infected with protozoa would have been possible.

Failure to establish how (and from which vector) the patient contracted the disease means that the reported case of babesiosis remains a mystery in terms of the epidemiology of the disease.

Regardless, the emergence of *B. gibsoni* in native dogs indicates that infections with this parasite should be taken into account and included in the differential diagnosis of vector-borne diseases. A diagnosis, as our own observations show, cannot be arrived at in a straightforward manner by examining stained blood smears. It is hard to distinguish small *Babesia* species when using staining methods; they also do not have a characteristic shape like *B. canis*, so the identification of invasion via light microscopy is unreliable. Therefore, when disease is suspected, it is advisable to perform a PCR assay with primers targetting *B. gibsoni* genes (Miro et al. 2015).

Clinical manifestations of *B. gibsoni* infections resemble those of *B. canis* (lymphadenopathy, enlargement of the spleen, diarrhoea, weight loss, proteinuria and nephropathy, haematuria and PU/PD). Laboratory studies show mild-to-severe regenerative haemolytic anaemia, neutropaenia and leukocytosis, as well as hypoalbuminaemia, azotemia and increased activity of liver enzymes (ALT, ALP). The course of the disease in most of the cases is severe (Macintire et al. 2002; Birkkenheuer et al. 2004; Lee et al. 2009). It is interesting that breeds such as Tosa Inu and the American Staffordshire terrier tend to be predisposed to the development of the discussed disease, and it tends to be more severe in these breeds. This may explain the difficulty in treating the patient (Birkkenheuer et al. 2004).

The treatment of *B. gibsoni* infection is much more difficult than the treatment of *B. canis* infection. Imidocarb may be used in the treatment of babesiosis, although it does not show efficacy against some small piroplasms (as in this case) (Solano-Gallego et al. 2016). In such cases, the alternative is a combination of atovaquone (13.5 mg/kg *p.o.* every eight hours with fatty food) and azithromycin (10 mg/kg *p.o.*) for ten days (Di Cicco et al. 2012). Unfortunately, this combination also fails in many cases (Sakuma et al. 2009; Di Cicco et al. 2012). Another alternative treatment regimen is the combination of clindamycin, diminazene and imidocarb (Lin et al. 2012). Although antibiotics do not completely eliminate piroplasms, they can alleviate the clinical symptoms of the disease (doxycycline) (Lin and Huang 2010). The recommended dose of doxycycline is 10 mg/kg/day *p.o.* Clindamycin, in turn (25 mg/kg *p.o.* twice a day for 14 days), reduces the symptoms of the disease as well as contributing to the improvement of laboratory results. In the treatment of *B. gibsoni* infection, a combination of antibiotics such as clindamycin

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(11 mg/kg every 12 hours *p.o.*), metronidazole (15 mg/kg every 12 hours *p.o.*), and doxycycline (5 mg/kg every 12 hours *p.o.*); or enrofloxacin (2.5 mg/kg *p.o.* 12 hours *p.o.*), metronidazole (5–15 mg/kg every 12 hours *p.o.*) and doxycycline (7–10 mg/kg every 12 hours *p.o.*) (Suzuki et al. 2007; Lin and Huang 2010) was also tried. However, the effectiveness of these combinations remains debatable.

In recent years in Europe, there has been an increase in the rate of transmission of diseases which were hitherto considered as exotic and atypical for the dogs of this continent. As regards babesiosis, it is important to realise that this is not just one disease, but a group of diseases caused by different species of protozoa. The accurate identification of the species of parasite that causes the infection is crucial for developing the correct treatment regimen and prognosis. The first case of *B. gibsoni* infection in a Polish dog indicates that babesiosis caused by small piroplasms should be considered in the differential diagnosis of vector diseases, and that these cases, due to climate changes in many parts of the world, including Poland, will most likely be noted more often.

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