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Co-infection with *Babesia canis* and *Babesia gibsoni* in a dog

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RESEARCH ARTICLE

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ABSTRACT

A four-year-old intact male Boxer, that had a history of travelling to Serbia, was referred for lethargy and anaemia. Shortly before the dog was referred, it was diagnosed twice with an infection with *Babesia canis* and was treated with imidocarb both times. A blood smear evaluation was indicative of the presence of intraerythrocytic piroplasms. After receiving inconclusive results regarding the type of piroplasm, the dog was diagnosed with simultaneous infections with *B. canis* and *Babesia gibsoni* via real-time polymerase chain reaction (rt-PCR) testing. The dog was treated with imidocarb, atovaquone and azithromycin, and in a follow-up examination, the PCR results were negative for *B. canis* and *B. gibsoni*. Several weeks later, the dog was presented again, and a PCR was positive for *B. gibsoni*. After atovaquone and azithromycin failed to eliminate the parasites, a therapy attempt using metronidazole, clindamycin and doxycycline was initiated. Six months after diagnosis, the treatment appeared successful in eliminating *B. gibsoni*. This case report describes the clinical findings of the co-infection and the initiated diagnostic and therapeutic approaches.

KEYWORDS

Babesia canis, *Babesia gibsoni*, dog, co-infection, Austria, Serbia

INTRODUCTION

Canine babesiosis is an emerging tick-borne disease in Europe, caused by intraerythrocytic protozoans of the genus *Babesia*. The predominant species in Europe are *Babesia canis* and *Babesia vogeli*, followed by the small piroplasm *Babesia gibsoni* (Leschnik, 2020).

B. canis is transmitted by the tick *Dermacentor reticulatus* and is mostly found in Central and Eastern Europe (Solano-Gallego and Baneth, 2011; Sonnberger et al., 2021). The infection can be categorised as uncomplicated or severe. The uncomplicated manifestation is characterised by fever, lethargy and anaemia, while the severe manifestation can lead to multi-organ involvement (Solano-Gallego et al., 2016). However, little is known about the epidemiology and the course of infection of *B. gibsoni*. Clinical signs are unspecific and range from lethargy and fever to discoloured urine and anaemia (Yao et al., 2014).

B. gibsoni is endemic in Southeast Asia and its prevalence in Europe is highly variable, with cases reported in Spain, Italy, the United Kingdom, Germany, Poland, Serbia, Romania and Croatia (Solano-Gallego and Baneth, 2011; Baneth, 2018; Teodorowski et al., 2020). The

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infection is known to be transmitted by two modes: directly from dog to dog or via tick bites from mainly *Haemaphysalis* spp. ticks (such as *H. longicornis* or *Haemaphysalis hystricis*), while a role for *Rhipicephalus sanguineus* as a vector has also been suggested (Pantchev et al., 2015; Jongejan et al., 2018; Leschnik, 2020). The role of *R. sanguineus* as a vector of *B. vogeli* is recognised but it is unlikely that this tick species plays an important role in the transmission of *B. gibsoni* (Jongejan et al., 2018). Therefore, it is suggested that outside the endemic areas for *B. gibsoni* in Asia, where its proven *Haemaphysalis* vectors are distributed, transmission among dogs occurs without a tick vector. If transmitted from dog to dog, the infection is transmitted vertically or via saliva, wounds or ingested blood. Often, fighting dogs are over-represented as a result of organised dog fights (Pantchev et al., 2015; Solano-Gallego et al., 2016). The non-vector-borne transmission route of *B. gibsoni* can include blood transfusion, especially if the blood is not tested by PCR prior to its use for this purpose (Pantchev et al., 2015).

Today, acute babesiosis caused by *Babesia canis* is a frequent diagnosis by veterinarians in Central Europe, whereas *B. gibsoni* is rarely diagnosed. Furthermore, to the best of the authors' knowledge, no similar case involving co-infection with *B. canis* and *B. gibsoni* in a dog has been reported; hence, we present clinical findings and therapeutic options of a dog infected by both parasites and present an overview of this rare case. In addition, to highlight the prevalence of infections with different *Babesia* species, we also present the laboratory PCR test results of samples submitted by Austrian veterinarians in the period from 2009 through 2016.

CASE HISTORY

A four-year-old intact male Boxer dog was diagnosed with an infection with *B. canis* in Jagodina, Serbia (Latitude: 43° 58' 37.67" N; Longitude: 21° 15' 40.36" E), in April 2020. The local veterinarian treated the dog twice with imidocarb dipropionate by intramuscular (IM) injection 14 days apart (2.5 mg kg⁻¹). After this anti-babesial treatment the dog recovered quickly. One month later, in May, the dog was taken back to Austria, where it again displayed anorexia and lethargy. The local veterinarian in Austria performed a complete blood count (CBC), which revealed a regenerative anaemia and thrombocytopenia. He suspected a relapse, so a PCR for *Babesia* spp. was performed, which was positive, but there was no testing for subspecies. For this reason, the dog was again treated with imidocarb dipropionate by subcutaneous (SC) injection (6.6 mg kg⁻¹). After this treatment the dog did not recover, and six days later he was again presented for anaemia, lethargy, weight loss and discoloured urine. The referring veterinarian suspected an immune-mediated haemolytic anaemia secondary to the *B. canis* infection and treated the dog with prednisolone, amoxicillin/clavulanic acid, doxycycline and vitamin K. As the dog showed no signs of improvement, it was referred to the University of Veterinary Medicine Vienna in June. The

patient was reported to be otherwise well with no previous medical problems.

Clinical and diagnostic findings

At the time of presentation at the University of Veterinary Medicine Vienna, the dog was moderately depressed and inactive. Notable physical examination abnormalities included a reduced body condition score of 3/9, moderately pale mucous membranes, tachycardia of 136 bpm, a bounding pulse and a systolic heart murmur of 2/6. No other abnormalities were noted.

Abnormalities in the CBC (haematology analyser Advia® 2120i) included severe regenerative anaemia, leukocytosis, neutrophilia with left shift, and lymphopenia. No autoagglutination was noted. The results are displayed in Table 1.

Abnormalities in serum biochemistry (Cobas® c501) included an elevated C-reactive protein concentration (123 mg L⁻¹, reference range <35 mg L⁻¹) and a mild hypoproteinaemia (5.6 g dL⁻¹, reference range 6–7.5 g dL⁻¹).

Microscopic evaluation of the blood smear was performed by a board-certified specialist in veterinary clinical pathology and revealed the presence of small piroplasms (Figs 1A and 1B) within the erythrocytes; additionally, the presence of large piroplasms (Fig. 1A, lower arrow) was suspected.

The existing diagnostic findings indicated a co-infection with *B. canis* and *Babesia gibsoni*. For confirmation, an in-house rt-PCR (Qube MDx real-time analyser) was performed as described by Teodorowski et al. (2020) and the result for *B. canis* infection was positive; however, for *B. gibsoni* the result was inconclusive. Because of these inconclusive results, a conventional PCR was performed at the Institute of Parasitology of the University of Veterinary

Table 1. Haematological parameters

Haematological parameters		Reference values
Erythrocytes (10 ⁶ µL ⁻¹)	1.79	5.5–8
Haematocrit (%)	14.7	37–55
Haemoglobin (g dL ⁻¹)	4.1	12–18
MCV (fl)	82.1	60–77
MCH (pg)	22.9	19–24
MCHC (g dL ⁻¹)	27.9	31–34
Reticulocytes/µL	676,262	6,000–15,000
Corrected reticulocyte count (%)	12.34	
Leukocytes/µL	22,934	6,000–15,000
Lymphocytes/µL	458.68	780–4,500
Thrombocytes × 10 ³ µL ⁻¹	71	150–500
Band cells/µL	1,146.7	0–500
Neutrophils/µL	19,723.24	3,300–11,250
Mononuclear leukocytes/µL	1,605.38	0–500
Eosinophils/µL	0.02	0–800
Basophils/µL	0.02	0–150

MCV: mean corpuscular volume; MCH: mean corpuscular haemoglobin; MCHC: mean corpuscular haemoglobin concentration.



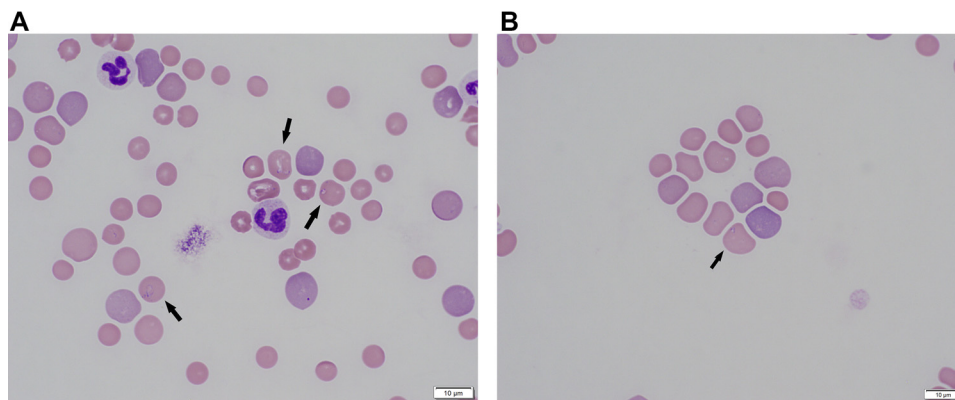


Fig. 1. Blood smear of the dog. A: *Babesia gibsoni* (upper arrows) and suspected *Babesia canis* (lower arrow). B: *Babesia gibsoni* (arrow) in an erythrocyte

Medicine Vienna; on this occasion the PCR result for *B. canis* was negative, but an all-Babesia-PCR was positive (*B. gibsoni*; GenBank ID: MZ293793). The all-Babesia-PCR was performed as described by Zintl et al. (2011), and a PCR for *B. canis* was performed as described by Zahler et al. (1998). The PCR and real-time PCR were performed using the dog's blood, which was collected on admission at the animal hospital and before any treatment (blood transfusion, imidocarb dipropionate) was initiated. An indirect fluorescent antibody test (IFAT) confirmed the presence of antibodies against *B. canis* (1:80).

Finally, to prove co-infection with *B. canis* and *B. gibsoni*, a real-time PCR was performed at IDEXX Laboratories using the dog's blood sample, which was collected from the referring veterinarian immediately before the first anti-babesial treatment in Austria. The sample was stored frozen at -20°C . The results were positive for *B. canis* (Ct value: 23.73) and *B. gibsoni* (Ct: value 25.44). The rt-PCR at IDEXX Laboratories, including the Ct values (reference range for positivity 6–39), was performed as follows: the total nucleic acid was extracted from an EDTA whole blood test by applying the QIAamp DNA Blood BioRobot MDx kit (QIAGEN, Germany) according to the manufacturer's instructions. A rt-PCR was performed using the LightCycler 480 (Roche) with proprietary forward and reverse primers and hydrolysis probes, taking a two-step approach: targeting the heat shock protein hsp 70 gene, the first rt-PCR assay detects *Babesia* spp., and in the case of a positive test result, species differentiation is achieved by individual rt-PCR assays specific for *B. canis* (hsp 70, gene accession number AB248735), *B. vogeli* (hsp 70, EF527401), *Babesia rossi* (hsp 70, AB248738), *B. gibsoni* (hsp 70, AB248731), and *Babesia conradae* (ITS2, AY965739).

PCRs for *Anaplasma phagocytophilum*, *Hepatozoon canis* and *Ehrlichia canis* and a PCR-restriction fragment length polymorphism (PCR-RFLP) for various *Babesia* spp. (including *B. vulpes*) were performed with blood taken on the day of presentation at the university, and all results were negative. The PCRs were performed as described previously

(Leschnik et al., 2012; Duscher et al., 2014; René-Martellet et al., 2013).

Therapy

The primary care veterinarian at the animal hospital began initial supportive therapy as follows: intravenous (IV) fluid therapy (Sterofundin $5\text{ mL kg}^{-1}\text{h}^{-1}$) and maropitant (1 mg kg^{-1} , IV). Due to the dog's unstable clinical condition owing to anaemia, weakness and tachycardia (136 bpm), blood typing was performed, which revealed blood type DEA 1, and the dog was treated with a single blood transfusion of 250 mL.

Due to the suspected large piroplasm and the dog's unstable clinical condition, a persistent infection with *B. canis* was suspected, so the dog was again treated with imidocarb dipropionate 6.6 mg kg^{-1} via intramuscular injection. Shortly thereafter, the dog displayed severe side effects, such as nausea and hypersalivation, bronchial hypersecretion and lethargy. The adverse effects were treated with atropine (3 boluses of $5\text{ }\mu\text{g kg}^{-1}$, IV).

One day after treatment for *B. canis*, the treatment for *B. gibsoni* was initiated as follows: the patient was started on 13.5 mg kg^{-1} atovaquone per os (PO) q8h and 10 mg kg^{-1} azithromycin PO q24h for 10 days. In total, the dog was hospitalised for two days, and although he was in moderate clinical condition, the owners refused its further hospitalisation but brought him back for regular follow-up examinations.

Two days after discharge the dog's condition improved, but he still displayed pale mucous membranes.

Within four weeks of discharge, the dog returned for several follow-up examinations. A physical examination revealed no abnormalities, a CBC revealed a slight thrombocytopenia, but serum biochemistry revealed no abnormalities and a PCR for *B. gibsoni* and *B. canis* was negative.

At the end of August, eight weeks after the end of therapy, the patient returned for another examination. A CBC revealed severe thrombocytopenia and the rt-PCR confirmed the presence of *B. gibsoni* (Ct value: 20.58).

Otherwise, the patient was in good clinical condition. Further treatment with atovaquone and azithromycin for 10 days was initiated, but this time the treatment seemed to fail in reducing the parasite load as on follow-up examination two weeks after the end of therapy, the dog still displayed thrombocytopenia and also displayed anaemia, and the result of the PCR was positive for *B. gibsoni*. The dog's condition deteriorated and, due to the failure of atovaquone and azithromycin, we started a treatment protocol with metronidazole (15 mg kg⁻¹ PO q12h), doxycycline (5 mg kg⁻¹ PO q12h) and clindamycin (2 mg kg⁻¹ PO q12h) for five weeks (Almendros et al., 2020). With this protocol, the dog's condition improved, and three months later he is still healthy, a CBC and serum biochemistry revealed no abnormalities, and a PCR and rt-PCR were negative for *B. gibsoni*. Figure 2 summarises the course of the CBC before and after the different treatment protocols.

Results of laboratory prevalence determined by the above-mentioned Babesia rt-PCR (IDEXX Laboratories) and conventional Babesia PCR (Veterinary University Vienna)

With the PCR assay targeting the heat shock protein hsp 70 gene, serum samples sent from Austrian veterinarians to IDEXX Laboratories between 2009 and 2016, mainly for the clinical suspicion of babesiosis or within tick-borne or travel disease profiles, were analysed (see Table 2). Within this tested population, the calculated probability of a simultaneous detection (co-infection) of *B. canis* and *B. gibsoni* is

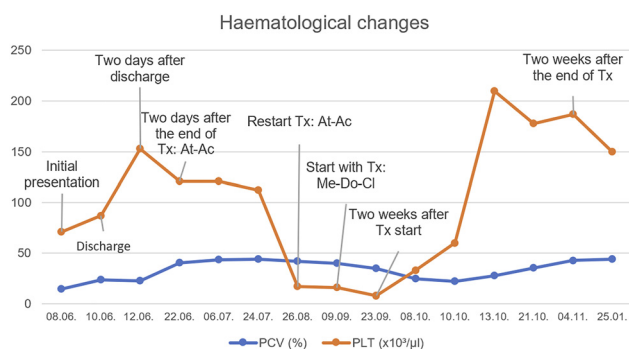


Fig. 2. Changes in haematological parameters in a timeline including treatment interventions. PCV: packed cell volume; PLT: platelet counts; Tx: Treatment; At: Atovaquone; Ac: Azithromycin; Me: Metronidazole; Do: Doxycycline; Cl: Clindamycin

Table 2. IDEXX *Babesia* real-time PCR results, 2009–2016 (screening with subsequent differentiation of the species) and results of conventional *Babesia canis* and piroplasm PCR from the Institute of Parasitology, Veterinary University Vienna (VetmeduniVienna), 2009–2020

Laboratory	PCR	n	Total positive, %	<i>B. canis</i>	<i>B. gibsoni</i>	<i>B. vogeli</i>	ND*
IDEXX	Multiplex-real-time	1,164	10.8%	90%	4%	3%	3%
VetmeduniVienna	<i>B. canis</i>	226	8.4%	100%	–	–	
	Piroplasm**	39	20.5%	62.5%	25%	12.5%	

*Not determined: differentiation not possible mainly due to low amount of nucleic acid.

**With subsequent sequencing.

1/2,356. Additionally, all PCR results for *Babesia* spp. between 2009 and 2020 from the Institute of Parasitology at the University of Veterinary Medicine Vienna, performed with conventional PCRs, are displayed in the same table, and the calculated probabilities for a co-infection are 1/152 (by piroplasm PCR) and 1/232 (by combined conventional PCRs).

DISCUSSION

This case raises three points of particular interest.

The first point is the simultaneous infection by *B. canis* and *B. gibsoni*. To the best of our knowledge, this is the only reported case of a dog presenting with a co-infection of *B. canis* and *B. gibsoni*. The severe anaemia and severe clinical condition may have been the result of infection by both parasites, but although the infection in our patient seemed to be severe, no organ involvement was detected (Solano-Gallego et al., 2016). According to the Ct values, the parasite burden of *B. canis* and *B. gibsoni* in our patient was relatively increased as Ct values of approximately 20 indicate a high parasite load; hence, there appears to be no correlation between the level of parasitaemia and the severity of anaemia (Furlanello et al., 2005). Nonetheless, Ct values might be tools for monitoring treatment response. Regarding *B. canis*, different strains in Europe with possibly variable pathogenicity, based on Bc28.1 gene polymorphism, have been documented (A, B and 34.01/G⁻¹) by RFLP (Carcy et al., 2015). Using another strain classification based on the 18S rRNA gene of *B. canis*, proteomic analysis of the serum of dogs revealed three different genotypes (A, B and C) among dogs within a single country (Poland; Lyp et al., 2015). These genotypes were assigned to either subclinical (C), acute (B) or mild (A) clinical courses. The Bc28.1-A strains were found to be either 18SrDNA-A or 18SrDNA-B; however, Bc28.1-B strains ($n = 9$) were all 18SrDNA-B (Carcy et al., 2015). Nevertheless, it should be noted that a different clinical condition in a dog could be attributed to factors other than a different *B. canis* strain alone, such as a co-infection or other underlying disease, tick prophylaxis, dog breed or usage (i.e. hunting, outdoor), age/sex, season and region (i.e. tick vector frequency), among others (René-Martellet et al., 2013, 2015). To date, there have been no further reports of *Babesia* co-infections except for a co-infection with *B. canis* and *B. vulpes* that was reported in two dogs in Spain (Checa et al., 2019). The recent findings of



Babesia vulpes n. sp. (syn. *Theileria annae*, *Babesia annae*, *Babesia microti*-like, *Babesia* cf. *microti*, ‘*Babesia* Spanish dog isolate’) in foxes in Germany, Austria and Hungary (Farkas et al., 2015; Pantchev et al., 2015; Hodžić et al., 2018) are intriguing as no concurrent infections in dogs were reported for the corresponding areas. Reports also exist for other European countries, including Portugal, Croatia, Sweden, France and Serbia, suggesting that canine piroplasmosis caused by *B. vulpes* n. sp. is more frequent than initially thought (reviewed by Checa et al., 2019). In north-western Spain this small *Babesia* frequently causes disease in dogs, as described in 75 clinical cases by Miró et al. (2015). Like other small *Babesia*, *B. vulpes* n. sp. does not respond well to imidocarb treatment, but a comparable efficacy (to *B. gibsoni*) was achieved in dogs with a combination treatment of either atovaquone or buparvaquone with azithromycin (Checa et al., 2017).

The presumed area of infection for *B. canis* and *B. gibsoni* is Serbia. Several surveys show that both hosts, *D. reticulatus* and the suggested vector *R. sanguineus*, and both piroplasms can be found there (Davitkov et al., 2015; Potkonjak et al., 2016, 2020). Moreover, it is known that *B. canis* is endemic in Belgrade and *B. gibsoni* can be found in the area of Pancevo, both of which are approximately 100 km from Jagodina, the former residence of our patient (Gabielli et al., 2015; Janjić et al., 2019). The dog had no history of blood transfusions, and as the owner reported no previous dogfight involvement, thus there has been no treatment with an acaricide or repellent, a tick-borne transmission appears more probable, but a transplacental transmission cannot be excluded. As mentioned above, the vector role of *R. sanguineus* in the transmission of *B. gibsoni* has not been proven to date, which is why non-vector-transmission should also be considered.

The second point of particular interest regarding the case is the diagnosis and the different PCR results. The initial anti-babesial treatment in Serbia may have reduced the parasitaemia load of *B. canis*, but as imidocarb dipropionate had been underdosed (recommended dose: 6.6 mg kg⁻¹; Baneth, 2018), it appears to be insufficient for eliminating the parasites. The additional infection with *B. gibsoni* could have weakened the dog’s immune system, effecting another fulminant outbreak of acute babesiosis caused by *B. canis*.

Back in Austria the third treatment with imidocarb may have reduced the parasitaemia load of *B. canis* below the limit of detection of the PCR, and this may be the reason why the conventional PCR performed at the University was negative for *B. canis*. IM or SC injection of imidocarb is recommended to be repeated after two weeks. In this case, the single administration given by the local veterinarian could have been insufficient for eliminating *B. canis* completely (Baneth, 2018).

The results of the in-house real-time PCR compared to the conventional PCR were inconclusive, whereas the real-time PCR at IDEXX Laboratories revealed positive results for *B. canis* and *B. gibsoni*. Several authors (Wahlang et al., 2019; Kuo et al., 2020) have demonstrated that real-time PCR is more sensitive in diagnosing *B. gibsoni*, *B. canis* and

B. vogeli than conventional PCR, especially in detecting low-level parasitaemia as seen in chronic infections caused by different piroplasms. A conventional PCR using only one pair of primers can lead to amplification of only single species, whereas a multiplex (or individual) PCR with separate primer pairs and hydrolysis probe will reveal co-infection with different species. Although the small piroplasm had already been detected with light microscopy, for definitive diagnosis and adequate treatment, PCR for *Babesia* spp. should have been an integral part of the diagnostics due to aspects such as storage. Blood smear evaluation can lead to a false diagnosis as large babesiae can have a similar morphology as small babesiae (Demeter et al., 2011).

The positive IFAT indicates exposure to *Babesia* spp., but this is insensitive as there is significant cross-reactivity between antibodies and large and small piroplasms (Pantchev et al., 2015; Solano-Gallego et al., 2016).

Regarding the PCR results for *Babesia* spp. from IDEXX Laboratories between 2009 and 2016 and the Institute of Parasitology between 2009 and 2020 (Table 2), the most often identified species was *B. canis* (Pantchev et al., 2015; Globokar Vrhovec et al., 2017). A possible explanation is that a larger proportion of the animals with a history of travel were tested preventively irrespective of clinical signs (i.e., within travel disease profiles), whereas the animals that had stayed within Germany or Austria were tested based on clinical suspicion. Additionally, *B. canis* is already endemic in Austria (Leschnik, 2020; Sonnberger et al., 2021). Furthermore, *B. gibsoni* was frequently found in fighting dogs and dogs imported from Asia (i.e. Sri Lanka), and *B. vogeli* was detected in dogs imported from the Mediterranean region (Dyachenko et al., 2012; Pantchev et al., 2015). Based on these findings, in Austria and Germany in particular the risk of a co-infection with different species of *Babesia* in dogs appears to be quite low, and the same might be true for the rest of Central Europe. The probability of co-infection detection depends on the PCR protocol applied, as well as on the dog population tested.

The third point of interest relates to treatment. As described previously, the treatment of choice for *B. canis* is imidocarb dipropionate via IM or SC injection, which appears to be efficient in eliminating the parasites (Solano Gallego et al., 2016). The adverse effects of imidocarb include cholinergic overstimulation such as salivation and vomiting, and these effects were recorded for our patient (Baneth, 2018). In rare cases, anaphylactic reactions can have a fatal outcome (Dyachenko et al., 2012). Furthermore, occasional renal tubular necrosis and hepatotoxicity after treatment are also possible, so the application of appropriate fluid therapy and lowering the dose of the drug (e.g. 3 mg kg⁻¹) in patients suspected to have renal involvement have been suggested (reviewed by Dyachenko et al., 2012). In contrast, treating *B. gibsoni* can be challenging as the parasites are hard to eliminate, but the combination of atovaquone and azithromycin appears promising in reducing parasitaemia below the PCR detection limit. Kirk et al. (2017) found that even 60 days after treatment with



azithromycin and atovaquone, 90% of infected dogs were testing negative for *B. gibsoni* via PCR. What makes treatment challenging is resistance to atovaquone, which is associated with mutations in the genome of *B. gibsoni*, in particular the M128 position of cytochrome b (Birkenheuer et al., 2018). Matsuu et al. (2006) found that *B. gibsoni* collected from three dogs displayed resistance to atovaquone following previous therapy. The same could be said of our patient as an rt-PCR was positive even after two treatment episodes with atovaquone and azithromycin. For this reason, we started a therapy with metronidazole, doxycycline and clindamycin. Almendros et al. (2020) found that 87% of dogs treated with this protocol remained PCR negative for *B. gibsoni*, after the combination of atovaquone and azithromycin failed to eliminate *B. gibsoni*. The dog's condition improved under this therapy and it is now healthy, with its rt-PCR and PCR remaining negative for *B. gibsoni*.

Regarding the CBC, the thrombocytes in our patient were reduced from $153 \times 10^3 \mu\text{L}^{-1}$ to $112 \times 10^3 \mu\text{L}^{-1}$. The thrombocytopenia was noted on day 14 and worsened over time, discovered at follow-up examinations. Based on this finding, we presume that parasites are still present but below the PCR limit of detection, owing to one of the cardinal signs of babesiosis, caused by *B. canis* or *B. gibsoni*, being thrombocytopenia (Solano-Gallego et al., 2016). Further explanation for this finding could be the presence of immune-mediated platelet destruction or platelet sequestration in the spleen (Barić Rafaj et al., 2013).

Infection with *Babesia* spp. is an important differential diagnosis for evaluating patients with anaemia, thrombocytopenia and a history of travel in endemic areas. More dogs may be suffering from co-infections with different piroplasms, but when the level of parasitaemia is low, it can be challenging to obtain a definitive diagnosis by conventional PCR, especially in infections with *B. gibsoni*. In this case, species-specific real-time PCR performed either as a multiplex or as an individual reaction could be superior to conventional PCR technique.

Notably, performing a regular screening PCR because co-infection is suspected, where the *B. gibsoni* parasitaemia is low, can result in positive PCR results purely for *B. canis*. In this case, the infection with *B. gibsoni* can be easily overlooked; thus, an rt-PCR as a first-screening reaction with subsequent species-specific PCRs for differentiation of the detected *Babesia* species can comprise an effective diagnostic method.

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