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Short communication

Repeated imidocarb treatment failure suggesting emerging resistance of *Babesia canis* in a new endemic area in north-eastern Germany

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ABSTRACT

Canine babesiosis has been increasingly diagnosed in various regions of Germany such as north-eastern Germany in recent years. A dog with several relapses of *Babesia canis* infection after treatment with imidocarb is described. A 9-year-old male Magyar Viszla with *B. canis* infection was referred after two treatments with imidocarb (dosage 2.1 mg/kg SC) because of lethargy, fever and pancytopenia (additional treatments with prednisolone and doxycycline). Merozoites were detected in the blood smear and imidocarb treatment was repeated. Clinical signs, pancytopenia and a positive *B. canis* PCR occurred after the 3rd (6 mg/kg SC), 4th (7.7 mg/kg SC) and 5th (7.5 mg/kg SC and doxycycline for 4 weeks in addition) imidocarb injection and thorough tick prevention with isoxazoline and permethrin products. 12 days after the 5th injection, the PCR was negative for the first time. The dog was again presented with fever 35 days after the 5th injection. The *B. canis* PCR was positive and laboratory examination revealed pancytopenia. Treatment with atovaquone/azithromycin for 18 days was performed and no further relapse occurred for 32 weeks. In the case of suspected imidocarb resistance in *B. canis* infection, treatment with atovaquone/azithromycin can be an alternative.

1. Introduction

The vector of *Babesia canis, Dermacentor reticulatus*, is increasingly spreading in Europe (Birkenheuer et al., 2020; Rubel et al., 2020, 2022). Babesiosis is therefore also referred to as an "emerging infectious disease" in veterinary medicine and the vector is highly abundant in the area of Berlin/Brandenburg in the vegetation as well as on dogs (Kohn et al., 2019; Bajer et al., 2022; Pawelczyk et al., 2022; Weingart et al., 2023). The clinical signs and the course of disease are determined by the host's immune system, the presence of co-infections with other vector-borne diseases, and the *Babesia* species (Birkenheuer, 2023). According to the morphology of the protozoan, *Babesia* species are classified in large (e.g. *B. canis* and *Babesia vogeli*) and small forms (e.g. *Babesia gibsoni* and *Babesia vulpes*) (Solano-Gallego et al., 2016). In Europe, *B. canis* dominates. In southern Europe, *B. vogeli* and in north-eastern Europe, *B. gibsoni* are also detected (Birkenheuer et al., 2020). While dogs with *B. vogeli* infection usually have only mild or no clinical signs, infection with *B. canis* is often accompanied by severe clinical signs (Garcia-Quesada et al., 2021; Weingart et al., 2023). Imidocarb is the first-line treatment for infection with large *Babesia* species, while the small *Babesia* forms respond poorly to imidocarb (Baneth, 2018). Resistance to therapy of protozoan infection (e.g. *B. gibsoni* [dog], *B. canis* [dog], *Babesia microti* [human], *Theileria annulata* [cattle], and *Leishmania infantum* [dog]) is increasingly described not only in humans but also in veterinary medicine (Zygner et al., 2005; Liu et al., 2016; Goncalves et al., 2021; Marcos et al., 2022; Ali et al., 2022; Rogers et al., 2023). Pre-treatment with antiprotozoal agents and the emergence of mutations are discussed as possible mechanisms of resistance development (de Koning, 2017).

In the following, the history, clinical findings, laboratory abnormalities, therapy and the course of disease in a dog with *B. canis* infection and suspected imidocarb resistance are described.

2. Case description

A 9-year-old male castrated Magyar Viszla with fever and pancytopenia (leukocytes 3.4 $\times$ 10^9/L, platelets 17$\times$$10^9$/L, and hematocrit [Hct] 0.38 L/L) was diagnosed with *B. canis* infection by polymerase chain reaction (PCR) by the referring veterinarian in January 2023 and treated with imidocarb (Carbesia®, Intervet, Beaucouze, France) at a

Abbreviations: CRP, C-reactive protein; Hct, hematocrit; PCR, polymerase chain reaction.

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dose of 2.1 mg/kg 2 times in a 14-day interval (days 0 and 14). Although the vector for *Ehrlichia canis* is not endemic in Germany, the PCR test was initiated (negative result). Further PCR tests for infectious diseases (*Anaplasma phagocytophilum, Mycoplasma hemocanis*, and *Mycoplasma hematoparvum*) were negative. Laboratory values and general condition improved after the first injection. Five days after the 2nd injection (day 19), fever occurred again. Laboratory examination revealed pancytopenia (leukocytes 2.7 × 10^9/L, platelets 33×10^9/L, and Hct 0.35 L/L). The referring veterinarian suspected an immune-mediated pathogenesis and treated the dog with prednisolone (on day 8 after first presentation: 1.1 mg/kg initially, then 0.7 mg/kg q24h PO and then a tapering dosage until day 19) and doxycycline (6.25 mg/kg q12h PO for 2 days). As the clinical signs did not improve (fever up to 40.0 °C) and the dog still had pancytopenia, the dog was treated once with 1.4 mg/kg prednisolone orally and referred to the Small Animal Clinic, Freie Universität Berlin (day 23). The dog came from a breeder in Germany, is living in Potsdam (Brandenburg), and was regularly dewormed and vaccinated. Two years before, he had been taken abroad to Denmark and Austria. Before presentation to the private veterinarian the dog had been wearing a collar with flumethrin and imidacloprid for protection against ectoparasites for approximately 6 months.

On first presentation to the Small Animal Clinic, the rectal temperature was 38.2 °C. The dog was lethargic, mucous membranes were pale pink with a normal capillary refill time. The pulse was strong and regular (rate 112/minute). The respiratory rate was 36 breaths per minute. Palpation of the abdomen and peripheral lymph nodes were unremarkable. Hematological examination (Sysmex XT2000i, Sysmex Deutschland GmbH, Norderstedt, Germany) revealed severe thrombocytopenia (6 × 10^9/L) and moderate anemia (Hct 0.29 L/L) (Table 1). Blood smear evaluation (LT-SYS Haema rapid staining, Labor + Technik, Berlin, Germany) identified merozoites in a few erythrocytes. No platelet aggregates were seen, the number of platelets was 0–1 platelets per high power field. Abnormal clinicopathological findings (Konelab 60i, Thermo Electron GmbH, Dreieich, Germany) were an elevated C-reactive protein (CRP) concentration and an elevation of liver enzyme activities (AP and ALT) (Table 1). Radiographic examination of the thorax did not reveal any abnormal findings. Ultrasound examination of the abdomen (Logic S7, Scil animal care company GmbH, Viernheim, Germany) revealed splenomegaly with a homogeneous parenchyma. The dog was treated again with imidocarb (6 mg/kg SC, 3rd injection; day 23) and was hospitalized. A painful swelling occurred at the injection site. In addition, the dog received a balanced crystalloid solution (Sterofundin Iso®, Braun, Melsungen, Germany, 40 ml/kg/day IV). Within 3 days, the dog became asymptomatic and the laboratory values improved (Hct 0.30 L/L, platelets 70×10^9/L, and CRP 55.9 mg/L), so that he was discharged. Four days later, the laboratory values normalized. Eleven days after the 3rd injection (day 34), the dog was dull, had fever (39.5 °C) and pancytopenia (leukocytes 3.5 × 10^9/L, no platelets, and Hct 0.32 L/L). Again, merozoites were detected in the blood smear, and the *B. canis* PCR was positive (Laboklin, Bad Kissingen, Germany) (Fig. 1). The CRP concentration was elevated with 59 mg/L. The dog was hospitalized and treated symptomatically (fluid therapy and metamizole [Novacen®, CP-Pharma, Burgdorf, Germany, 20 mg/kg IV]). The administered imidocarb dosage was 7.7 mg/kg SC (4th injection; day 34). Due to a suspected resistance against imidocarb the dosage was increased. Similar dosages of imidocarb were given in a study of Penzhorn et al. (1995). No side effects occurred. *Babesia canis* antibody titer (IFAT, Megafluo *Babesia canis* ad us.vet.®, Megacor, Austria) was positive (> 1:64) (Chair for Experimental Parasitology, LMU Munich, Germany). The tests for anti-erythrocyte (Coombs' test) and platelet-bound antibodies were negative (Immunology Unit, University of Veterinary Medicine, Hannover, Germany). After initial normalization of the laboratory values, pancytopenia (leukocytes 2.3 × 10^9/L, platelets 60×10^9/L, and Hct 0.36 L/L) reappeared 10 days after the 4th injection (day 44). The rectal temperature of 38.8 °C was within the normal range but higher than usual for this dog (37.9–38.3 °C). Merozoites were not detected in the blood smear, but the *B. canis* PCR was positive again. In addition to symptomatic therapy (fluid therapy), imidocarb (at a dose of 7.5 mg/kg SC) was administered again. Side effects (hypersalivation, restlessness) occurred in connection with the 5th injection. Systolic blood pressure (Doppler, Eickemeyer, Tuttlingen, Germany) was between 120 and 130 mmHg, the heart rate was between 90 and 100 beats per minute. Symptomatic treatment was initiated (fluid therapy [50 ml/kg/24 h] and maropitant [1 mg/kg IV, Emex®, CP Pharma, Burgdorf, Germany]). Doxycycline (Doxybactin®, Dechra, Aulendorf, Germany; 5 mg/kg 2 q12h PO) was added for 4 weeks to the imidocarb treatment. The dog became asymptomatic, laboratory values normalized, and PCR testing for *B. canis* was negative for the first time (12, 16 and 22 days after the 5th imidocarb injection). 35 days after the 5th injection (day 79), the owner noticed a slightly higher rectal temperature (38.8 °C), laboratory examination revealed pancytopenia (leukocytes 3.25 x 10^9/L, platelets 82×10^9/L, and Hct 0.40 L/L) and CRP elevation (40.1 mg/L). Blood smear was negative, but PCR test for *B. canis* was positive again. Therapy was started with atovaquone (Wellvone Suspension®, GlaxoSmithKline GmbH & Co. KG, Berlin, Germany, 13.3 mg/kg q8/h PO with a high-fat meal) and azithromycin (Zithromax®, Pfizer Pharma GmbH, Berlin, Germany, 10 mg/kg q24/h PO). Both drugs were administered for 18 days (days 79 - 97) and no side effects occurred. The laboratory values normalized within 3 days. During the atovaquone/azithromycin treatment PCR was negative on 2 different dates (days 5 and 15 after starting), and continued to be negative 13 weeks (days 10, 16, 22, 29, 37, 44, 61, 74, 93) after discontinuation of treatment (Fig. 1). No further relapse was detected up to 32 weeks after the end of therapy.

Table 1
Clinicopathological findings in a 9-year old dog with *Babesia canis* infection and suspected imidocarb resistance at the day of referral to the Small Animal Clinic, Freie Universität Berlin (day 23).

Parameter	Patient	Reference range
Hematology		
Leukocytes (x 10^9/L)	7.2	5.6–14
Band neutrophils (x 10^9/L)	0	0–0.5
Segmented neutrophils (x 10^9/L)	4.5	3.0–9.0
Eosinophils (x 10^9/L)	0	0–0.6
Lymphocytes (x 10^9/L)	1.8	1–3.6
Monocytes (x 10^9/L)	0.9*	0–0.5
Hematocrit (L/L)	0.29*	0.42–0.56
Hemoglobin (g/dL)	10*	14.6–19.8
Erythrocytes (x 10^{12}/L)	4.6*	5.9–8.3
Reticulocytes (/μL)	23, 000	>60, 000+
Platelets (10^9/L)	6*	165–400
Clinical chemistry		
Sodium (mmol/L)	151*	140–150
Potassium (mmol/L)	4.3	3.6–4.8
Glucose (mmol/L)	5.6	4.5–6.2
Creatinine (μmol/L)	45*	54–123
Urea (mmol/L)	4.5	3.5–10
ALT (U/L)	108*	< 76
AP (U/L)	847*	< 97
AST (U/L)	22	< 41
Bilirubin (μmol/L)	3.9	< 5
Calcium (mmol/L)	2.3*	2.5–2.9
Phosphoros (mmol/L)	1.0	0.96–1.4
Protein (g/l)	57.6	54–66
Albumin (g/L)	28	28–36
Lipase (IU/L)	41	< 260
CRP (mg/L)	125*	< 10
Coagulation parameters		
aPTT (s)	13.1	10–13.1
PT (s)	24	16.5–25

* Abnormal values; + > 60, 000/μL is defined as regenerative anemia.

3. Discussion

This case report describes a dog with *B. canis* infection and treatment

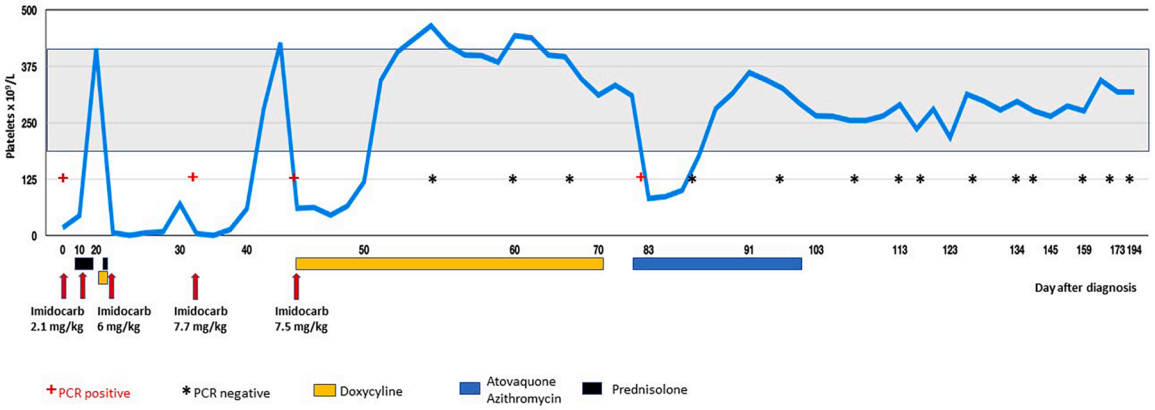

Fig. 1. Treatment and platelet counts in a 9-year-old dog with *Babesia canis* infection and suspected imidocarb resistance.

failure due to suspected imidocarb resistance. Imidocarb is the first-line treatment for infection with large *Babesia* species (Baneth, 2018). This antiprotozoal drug belongs to the carbanilids, whose mechanism of action is based on binding to the DNA of parasites with subsequent inhibition of nucleic acid synthesis. A reduced transport of inositol into the erythrocytes containing *Babesia* and subsequent death of the parasites due to lack of energy was also discussed (McHardy et al., 1986; Plumb, 2015). The manufacturer of Carbesia® gives different dosages for treatment of babesiosis: a therapeutic dose (2.125 mg/kg) and a preventive dose (4.25 mg/kg). The dosages are significantly lower than the recommended dosages in the literature, ranging from 5–7.5 mg/kg (Baneth, 2018; Birkenheuer, 2023). According to Birkenheuer, the differences in dosage recommendation are based on the fact that the treatment goal for dogs in endemic areas (where infection is very likely) is solely reduction in the number of babesia with subsequent formation of pre-immunity ("immunity of infection") (2023). Pre-immunity is defined as reduced morbidity in the event of reinfection in dogs with chronic subclinical babesia infections (Brandao et al., 2003; Sikorski et al., 2010). The patient described here was initially treated with 2.1 mg/kg imidocarb according to the instructions in the package insert. This could possibly explain the lack of response to therapy. The administration of such low dosages in *B. canis* infections should be questioned at least in non-endemic areas or areas where prevalence of *B. canis* is very low. In the present case it obviously not only failed to eliminate the parasites but also was not able to stabilize the dog to a level where its own immune response was able to control the disease and allows the development of pre-immunity. In this context, it cannot be excluded that the treatment with prednisolone given in context with the 2nd injection interfered with successful development of protective immune responses.

Different treatment protocols are described in the literature (studies, case reports): the number of injections ranges from 1 to 5, usually at an interval of 2 weeks (Penzhorn et al., 1995; Brandao et al., 2003; Boozer et al., 2003; Baneth, 2018; Seibert et al., 2022; Weingart et al., 2023). The dosages administered are also very variable in the literature, ranging from 2.1 to 7.5 mg/kg (Brandao et al., 2003; Sikorski et al., 2010; Eichenberger et al., 2016; Strobl et al., 2020; Bajer et al., 2022; Seibert et al., 2022; Weingart et al., 2023). In a recently published study, 49 autochthonous cases of *B. canis* infection diagnosed in the area Berlin/Brandenburg were evaluated (Weingart et al., 2023). Imidocarb was administered in most cases 2 times at a median interval of 15 days, the median dosage was 5 mg/kg (2.4 - 6.3). Eight dogs received only one injection (6 dogs died before the 2nd injection and one dog each went to the primary care veterinarian for further therapy or did not present for follow-up). Imidocarb was used with caution (lower dosage) in dogs with impaired renal and hepatic function (Kock and Kelly, 1991; Mathe et al., 2007; Plumb, 2015). After the first administration of imidocarb, *Babesia* PCR was still positive in 20 % of cases (7 of 35). A second injection of imidocarb was given to 38 dogs; 3 of them received 3, 4 and 5 injections, respectively, because the *Babesia* PCR was still positive after therapy. Dogs with a positive PCR test received a median imidocarb dosage of 4.3 mg/kg (3 - 6 mg/kg) and dogs with a negative PCR test received 5 mg/kg (4.3–6 mg/kg). The data from this study highlight the importance of routine PCR tests at approximately 2 weeks and 2 months after the end of therapy (Baneth, 2018).

The dog presented here received imidocarb subcutaneously because there was an increased risk of bleeding with an intramuscular injection due to severe thrombocytopenia. Described side effects of imidocarb include pain and swelling at the injection site (rarely ulcerative skin lesions) and cholinergic effects such as salivation, vomiting, diarrhea, panting, and restlessness. In rare cases, renal tubular damage and liver necrosis may occur (Kock and Kelly, 1991; Mathe et al., 2007; Plumb, 2015; Baneth, 2018).

Since a relapse occurred again after the third injection, a higher dose of imidocarb was administered and doxycycline was added to the treatment protocol after the 5th injection. Combination therapy with doxycycline, which is described in the literature, may lead to reduction in morbidity (Eichenberger et al., 2016; Strobl et al., 2020). In human medicine successful monotherapy with doxycycline (for 90 days) in a man with *Babesia venatorum* infection has recently been described (Huang et al., 2023). However, there are no confirmed data on the use of doxycycline alone for canine babesiosis therapy although it is regularly used as prophylaxis against other hematozoa, i.e. *Plasmodium* spp. in humans to prevent malaria (Vercammen et al., 1996a; Gaillard et al., 2015; Birkenheuer, 2023). Therapy of *B. canis* infection with doxycycline alone is therefore not recommended in dogs. The clinical practice guidelines for diagnosis and management of human babesiosis recommends to treat with either quinine or atovaquone, in combination with clindamycin or azithromycin. The antiparasitic activity of these antibiotics (clindamycin, azithromycin, and possibly also doxycycline) is based on protein synthesis inhibiting of the apicoplast, an organelle derived from a photosynthetically active ancestor, which is in the erythrocytic phase of haemosporidia predominantly required for synthesis of isoprenoid precursers (Krause et al., 2021; Buchanan et al., 2022; Huang et al., 2023).

A significantly longer interval until the 4th relapse (35 days) compared to the relapses before (5, 8 and 11 days after imidocarb injection) might have been due to the 28-day doxycycline therapy. After the 4th relapse, combination therapy with atovaquone and azithromycin was started. Atovaquone is a hydroxynaphtoquinone derivate used in human medicine for the treatment and prophylaxis of malaria and in veterinary medicine for the treatment of *B. gibsoni* and *B. vulpes* infections, but it is also effective against large *Babesia* species (Sikorski et al., 2010; Lalloo et al., 2016; Solano-Gallego et al., 2016; Baneth, 2018; Teodorowski et al., 2022; Birkenheuer, 2023). In a case series reported by Sikorski et al. (2010), one dog with a large *Babesia* spp.

infection according to blood smear examination was described. This dog was positive for a PCR detecting *Babesia* sensu stricto (including the canine parasites *B. canis, B. vogeli, Babesia rossi, B. gibsoni*, and *Rangelia vitalii* (Schnittger et al., 2022)) and negative in a *B. canis* -specific PCR. This dog was effectively treated with atovaquone and azithromycin. The reason for treating this dog with atovaquone/azithromycin was avoidance of side effects due to imidocarb treatment. The mechanism of action of atovaquone consists of a selective inhibition of the mitochondrial electron transport of the protozoa and a resulting inhibitory effect on pyrimidine synthesis (Plumb, 2015). According to recommendations in the literature, treatment with atovaquone/azithromycin for 10 days is recommended for *B. gibsoni* infection. The dog described here was treated for a total of 18 days. No side effects occurred. Different mechanisms of action of atovaquone/azithromycin compared to imidocarb was probably the reason for successful therapy.

Reinfection as a reason for the treatment failure was very unlikely since the dog received tick prophylaxis with sarolaner (Simparica®, Zoetis, Berlin, Germany), partly in combination with permethrin (Advantix®, Elanco GmbH, Cuxhafen, Germany) during the therapy. In addition, an experimental study found that dogs still had antibodies for five to eight months after infection and were protected against reinfection (Vercammen et al., 1996b). In the patient described here, the *B. canis* titer was > 1:64 34 days after diagnosis. Immunosuppression due to other co-infections or non-infectious diseases could not be detected in the patient described here. The influence of the prednisolone therapy administered by the pretreating veterinarian is unclear. Immunosuppressed patients are predisposed to *Babesia* infection, but therapy with imidocarb is effective in most of these patients (Sikorski et al., 2010; Seibert et al., 2022; Birkenheuer, 2023). In one study, 7 immunosuppressed dogs (6 with splenectomy, 1 with lymphoma under chemotherapy) with *Babesia* infection (large *Babesia* species) were described. Five dogs were treated with imidocarb (6.6 mg/kg [1 dog incorrectly received 3 mg/kg] 2 × 14–17 days apart). One dog received atovaquone/azithromycin (13.4 mg/kg q8/h PO / 10 mg/kg q24/h PO for 10 days) and another received no antiprotozoal therapy (Sikorski et al., 2010). Three dogs treated with imidocarb also received doxycycline and 1 dog azithromycin. Three dogs received glucocorticoids in addition to imidocarb. In all 7 dogs, the platelet counts normalized, 3 dogs remained anemic (1 dog without therapy and the other two dogs were euthanized due to intra-abdominal masses). PCR tests after treatment were performed in 5 dogs. The dog treated with 3 mg/kg imidocarb was 2 times PCR positive after 30 and 60 days. The dogs treated with 6.6 mg/kg imidocarb were negative in the PCR control, with one dog being diagnosed with babesiosis again after 340 days. The untreated dog remained PCR positive (Sikorski et al., 2010). In another case series with 81 dogs with *B. canis* infection 62 % of the cases were treated with prednisolone (2 mg/kg) for 3–4 weeks in addition to imidocarb (2.1–7 mg/kg 2 x at 14-day intervals) (Seibert et al., 2022). In 3 of 37 cases, a positive PCR test was detected 2 weeks after the 2nd imidocarb injection. Two dogs received 4 imidocarb injections, whereby one dog again had a positive PCR after the 4th injection. Further therapy was refused by the owner. The dose of imidocarb administered is not mentioned for this case. It is possible that the parasite population within this dog was also resistant to imidocarb. The other dogs in this study responded well to imidocarb therapy. Nevertheless, routine therapy with glucocorticoids is contraindicated in patients with babesiosis. Only in rare cases with associated immune-mediated hemolytic anemia (positive Coombs test, numerous spherocytes, and autoagglutination) and lack of hematocrit stabilization and in patients with bleeding due to severe thrombocytopenia, prednisolone therapy can be considered (Swann et al., 2019; Weingart et al., 2023).

4. Conclusion

Imidocarb can be administered subcutaneously and intramuscularly (Baneth, 2018). Imidocarb is the first-line treatment for *B. canis* infection. An adequate dose should be used, with consideration of the renal and liver function. The present data show that treatment of individual dogs with imidocarb can completely fail and the most likely explanation for this phenomenon is imidocarb resistance. However, in the absence of available in vitro tests to evaluate resistance and without performing experimental infections with potentially resistant isolates in multiple dogs to perform treatment experiments under controlled conditions it is difficult to formally prove imidocarb resistance. Treatment failure can potentially also be explained by characteristics of individual dogs such as increased metabolism or excretion of a drug or decreased drug uptake/systemic distribution. PCR tests should be performed routinely for treatment monitoring. In case of recurrence or suspected failure of imidocarb treatment, the use of atovaquone and azithromycin could be an alternative therapy.

Author statement

All authors have contributed to the work and agree with presented findings.

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C. Weingart and B. Kohn declare that they have held lectures for veterinary pharmaceutical and diagnostic companies. B. Kohn declares that she has acted as consultant for veterinary pharmaceutical and diagnostic companies and had research collaborations with various companies. J. Krücken declares no conflicts of interest.

CRediT authorship contribution statement

Christiane Weingart: Writing – review & editing, Writing – original draft, Visualization, Methodology, Formal analysis, Data curation, Conceptualization. **Jürgen Krücken:** Writing – review & editing, Writing – original draft. **Barbara Kohn:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Conceptualization.

Declaration of competing interest

C. Weingart and B. Kohn declare that they have held lectures for veterinary pharmaceutical and diagnostic companies. B. Kohn declares that she has acted as consultant for veterinary pharmaceutical and diagnostic companies and had research collaborations with various companies. J. Krücken declares no conflicts of interest.

Data availability

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