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Clinical, morphological, and molecular characterization of an undetermined *Babesia* species in a maned wolf (*Chrysocyon brachyurus*)

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ABSTRACT

A possible novel *Babesia* species infection of a maned wolf *(Chrysocyon brachyurus)* was first reported in 2012. The current case details a confirmed report of a maned wolf with infection by an undetermined species of *Babesia*. As the mortality and morbidity of babesiosis is high, this may become a significant concern to captive maned wolves, which are considered a near-threatened species by the World Association of Zoos and Aquariums. The aim of this study is to report the clinical, morphological and molecular characterization of this *Babesia* species. A 2.5-year-old, intact female maned wolf was found laterally recumbent with pale mucous membranes and jaundice the morning of presentation. Hematological and serum biochemical data were consistent with babesiosis and showed a regenerative severe anemia, leukocytosis, thrombocytopenia, hyperbilirubinemia, azotemia, increased creatine phosphokinase and increase alanine aminotransferase. On blood film review, inclusion bodies were seen in the red blood cells with cytomorphological features that were most consistent with a small form *Babesia* species. A blood sample was sent for polymerase chain reaction (PCR) testing and multi-locus sequence analyses. These findings suggested a unique *Babesia* species that is most closely related to a *Babesia* species (*Babesia* sp. AJB-2006) that has been found to infect raccoons (Procyon lotor) in North America. Although the cytomorphological features of the piroplasms and the clinical presentation were similar in both the current and 2012 case, when comparing the 18S melt curve temperature of the two *Babesia* isolates, the peak temperature was different. Unfortunately, genetic material from the 2012 case was not available so comparison of multi-locus gene sequences could not be performed, excluding the possibility to definitively state if the *Babesia* spp. from both cases were distinct from each other. The maned wolf was treated with a whole blood transfusion, dexamethazone (0.28 mg/kg IM), azithromycin (10 mg/kg in NaCl SC), atavaquone (1.5 cc PO), and 2 imidocarb (6.6 mg/kg IM) injections, and clinically improved. These findings demonstrate the need to further characterize the molecular and epidemiological differences of the *Babesia* species in this case report and the *Babesia* species known to infect raccoons.

1. Introduction

The wild population of maned wolves (*Chrysocyon brachyurus)* is decreasing as a result of habitat loss to agricultural practices (de Almeida Curi et al., 2011). Thirty years ago the Association of Zoos and Aquariums included the maned wolf as part of the Species Survival Park Program in order to try and keep a viable and healthy population of maned wolves and prevent the loss of this population (Songsasen and Rodden, 2010). Thus, as more cases of tick-borne pathogens are seen in maned wolves, it represents a significant concern to their conservation.

The first case report of a maned wolf infected by *Babesia* was documented by Phair et al. (2012). Interestingly the wolf from this previous case report and the wolf from the present case report originated from the same zoological park in Kansas. Unfortunately the *Babesia* species in the previous case could not be definitively identified but a novel species of *Babesia* was suspected due to equivocal PCR findings.

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The aim of this study was to characterize the clinical, morphological and molecular features of a unique *Babesia* species in a maned wolf.

2. Case presentation

2.1. Anamnesis

A 2.5-year-old female intact maned wolf from a regional zoological park was presented for emergency evaluation to the Zoological Medicine Service at Kansas State University's Veterinary Health Center. The wolf was found laterally recumbent in her crate on the morning of presentation with evidence of watery diarrhea. The wolf had a 3-day history of decreased appetite, lethargy and ataxia.

2.2. Clinical signs and clinical examination results

On physical examination, the wolf had a body condition score of 2/5. Mucous membranes were tacky, pale and mildly icteric with a prolonged capillary refill time (CRT) of 3 s. Dehydration was estimated to be 8–10% with weak femoral pulses. Brown diarrhea was presented in the rectum. An unidentified tick was found crawling on the wolf's fur. Unfortunately, the tick was lost before it could be identified. Blood and urine were collected for analysis (Supplementary Appendix A.1).

3. Material and methods

3.1. Molecular analyses

3.1.1. Samples and DNA isolation

Blood samples were available from the maned wolf in this study as well as from a raccoon that was previously confirmed to be solely infected with *Babesia* sp. AJB-2006 (Birkenheuer et al., 2006). For both samples, DNA was isolated from 200 µL of whole blood using a commercially available kit according to manufacturer's instructions (QIAamp DNeasy Blood and Tissue Kit, Qiagen, Valencia, CA).

3.1.2. PCR primers and controls

All PCR primers utilized in this study are summarized in Table 1 and in Supplementary Table A.1. Positive controls for all PCRs consisted of DNA extracted from *Babesia*-infected canine whole blood; negative controls consisted of DNA extracted from non-infected canine whole blood and of water (no DNA). Protocols for each individual PCR are outlined as follows.

3.2. Commercial molecular diagnostic testing

To initially confirm if the observed parasites were *Babesia* organisms, the maned wolf DNA sample was subjected to a broad range *Babesia* PCR as well as multiple species-specific *Babesia* PCRs as previously described (Birkenheuer et al., 2003). The assays were modified for a quantitative real-time PCR platform (Vector Borne Disease Diagnostic Laboratory, North Carolina State University, College of Veterinary Medicine). Cycling conditions were as follows: denaturing phase at 98 °C for 3 min followed by 40 cycles of 98 °C for 15 s, 60–62 °C for 15 s, and 72 °C for 20 s with a plate read following each cycle. All melt curve analyses were as follows: 65 °C–95 °C in 0.5 °C increments with a 2 s pause between phases for plate reading.

For comparison to the results of the previously described maned wolf with babesiosis (Phair et al., 2012), *Babesia* 18S rRNA was amplified and assessed through use of quantitative FRET-PCR and melt curve analysis as previously described (Wang et al., 2010; Molecular Diagnostic Laboratory, Auburn University, College of Veterinary Medicine). Samples infected with *Babesia* species that commonly infect domestic dogs were assessed in tandem with the maned wolf sample for melt curve comparison.

Additional molecular analyses, including multi-locus gene (18S rRNA, cytochrome c oxidase subunit I (cox1, Schreeg et al., 2016), and beta-tubulin) PCR, sequencing, and phylogenetic analysis are detailed in Supplementary A.2.

Table 1
Sequences of primers used in this study.

Primer	Sequence (5'-3')	Reaction and/or Use
Btub Frag1F	TGTGGTAACCATATYGGWGCCA	Beta tubulin fragment 1 forward primer
Btub Frag1R	CCGTGGTAGGTTCCGCTCT	Beta tubulin fragment 1 reverse primer
Btub Frag2F	CATCTCTGACGAGCATGG	Beta tubulin fragment 2 forward primer
Btub Frag2R	CGGTRTARTGMCCYTTRGCCCA	Beta tubulin fragment 2 reverse primer

4. Results

4.1. Laboratory analysis

Abnormalities on serum biochemistry analysis included hyperbilirubinemia, increased alanine aminotransferase (ALT), creatine phosphokinase (CK), blood urea nitrogen (BUN), hypoalbuminemia and hypokalemia (Supplementary Table A.2). No significant abnormalities were identified in the urine sediment. Snap 4DX Plus Test was negative. CBC abnormalities included a regenerative severe anemia, neutrophilia with left shift, and severe thrombocytopenia (Supplementary Table A.3). On blood film review, occasional rubricytes, metarubricytes and a moderate to high density of polychromatophils were observed. Rare spherocytes and schistocytes were seen. Numerous erythrocytes (~90% of erythrocytes) contained one to rarely two, small, variably shaped intraerythrocytic piroplasms (Fig. 1). Based on measurement of 20 organisms, length varied from 2 to 7 µm (mean = 4.8 ± 1.3 µm) and width varied from 1 to 5 µm (mean = 2.9 ± 1.2 µm). Piroplasm morphology was most often round to signet ring-shaped, but occasionally elongated, with punctate nuclei. Rarely, these organisms were seen free in the background. The cytomorphologic features of the organisms were most consistent with a small form *Babesia species*. A saline agglutination test was positive up to a dilution of 1:9, which indicated an immune-

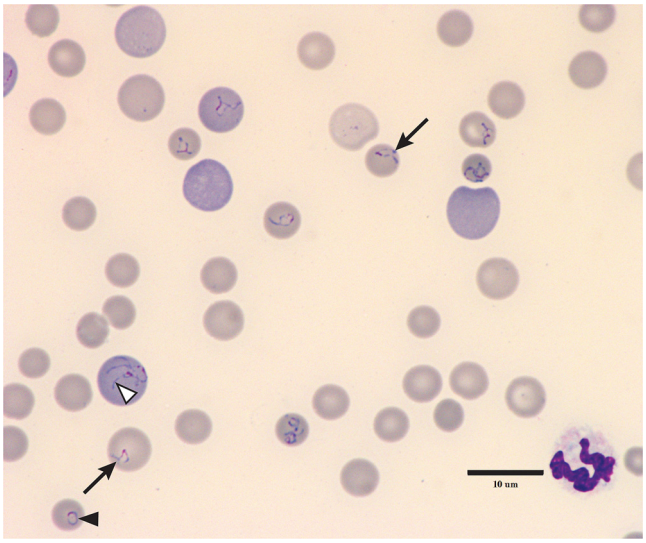

Fig. 1. Blood film from a maned wolf. Note the small (1–2.5 µm in length) signet ringed (arrow), round (full arrow head) and elongated (empty arrow head) piroplasms with punctuate to peripheralized nuclear material in multiple erythrocytes. Increased macrocytes (polychromatophils) and moderate spherocytosis is also present. Modified- Wright's stain. Bar = 10 µm.

mediate component to the anemia, supported by the presence of spherocytosis.

4.2. Molecular analyses

Initial screening by a commercial diagnostic laboratory (Vector Borne Disease Diagnostic Laboratory, North Carolina State University, College of Veterinary Medicine) determined that blood obtained from the maned wolf was positive for *Babesia* DNA using a broad range PCR that detects most *Babesia* species, but was negative when using specific assays that detect *Babesia* species that commonly infect domestic and wild canids (Table 1). Furthermore, when analyzed by an additional commercial diagnostic lab (Molecular Diagnostic Laboratory, Auburn University College of Veterinary Medicine) using FRET-PCR, the melting peak temperature of the amplicon produced from the maned wolf *Babesia* sp. (59.6 °C) was distinct from that of the controls [*B. gibsoni* (61.4 °C), *B. rossi* (52.6 °C), and *B. canis/vogeli* (58.7 °C)].

Given the inability of commercially available assays to identify the *Babesia* species infecting the maned wolf, multi-locus gene sequencing of the parasite was performed. Sequences of all three genes amplified (18S rRNA, *cox1*, and beta-tubulin) were most similar to that of the previously described *Babesia* sp. AJB-2006, which was identified in samples from raccoons in North America (Birkenheuer et al., 2006). In comparing the two species, 18S rRNA sequences shared 99.8% nucleotide identity, *cox1* sequences shared 98.1% nucleotide identity and 100% amino acid identity, and beta-tubulin sequences shared 98.2% nucleotide identity and 100% amino acid identity. Phylogenetic analysis of nucleotide (18S, beta-tubulin, and *cox1*) and amino acid (*cox1*) sequence grouped the *Babesia* species from the maned wolf in this report and the *Babesia* species from North American raccoons in single clade within the larger clade of "true" *Babesia* species (AKA *Babesia* sensu stricto; Supplementary Figures B.1–3).

4.3. Clinical outcome and treatment

The maned wolf received a whole blood transfusion collected from its pen mate. Initial therapeutic intervention during 4 days of hospitalization included dexamethasone sodium phosphate (0.28 mg/kg once per day [SID] delivered intramuscularly [IM]), vitamin B complex (0.5 cc in subcutaneous fluid), and multimodal antibiotic therapy, including oxytetracycline (20 mg/kg SID IM) and single dose of enrofloxacin (6.4 mg/kg diluted in 30 cc LRS, administered subcutaneously [SC]). After 4 days of hospitalization, the wolf was returned to the zoo and all drugs were given *per os* in food, including 11 days of azithromycin (10 mg/kg SID *per os* (PO), for 11 days), 8 days of minocycline (11.6 mg/kg SID PO), and 10 days of atovaquone (13.3 mg/kg SID PO). Fipronil and (S)-methoprene (Frontline Plus 45-88#, 3239 Satellite Blvd., Duluth, GA) was applied topically per manufacturer instructions for flea and tick prevention. In addition, the wolf received two doses of imidocarb dipropionate (6.6 mg/kg IM), with a gap of 2.5 weeks between injections.

After thirty-days, clinical re-evaluation showed all physical examination, CBC, and serum biochemistry findings within normal limits. No organisms were visible on repeat blood film inspection and PCR testing was negative for *Babesia*.

5. Discussion

The maned wolf in this case was presented with similar clinical signs as the maned wolf from the previous case report, which was the first indicator for a possible infection by *Babesia*. This case appeared to respond to treatment with a combination of antibiotics and anti-protozoal drugs. It is not clear which treatments, if any, were responsible for the clinical response.

In this case, the suspicion of babesiosis was confirmed by a blood film review, in which a high degree of parasitemia due to a small form *Babesia* species was identified. While the organisms described in both this report and the previous were cytomorphologically similar (small form *Babesia* sp.), 18S melt curve analysis indicated that the *Babesia* species described in this study (peak melting temperature of 59.6 °C) was distinct from *Babesia* species that commonly infect domestic dogs (*B. gibsoni* and *B. canis* species) as well as the *Babesia* species previously identified in a maned wolf (peak melting temperature of 58.8 °C) (Phair et al., 2012). Sequence characterization of three genes (18S rRNA, *cox1*, and beta-tubulin) indicates that this organism is a "true" *Babesia* (*Babesia* sensu stricto) and was most closely related to a *Babesia* species that infects raccoons in North America (*Babesia* sp. AJB-2006; Supplementary Fig. B.1–3). Multi-locus gene sequencing would be the ideal method to determine whether or not organisms from this case and the 2012 case are identical or different species of *Babesia*. Unfortunately the absence of genetic material from the original case precludes this comparison and thus excluded the possibility to definitively state if the *Babesia* spp. from both cases were distinct from each other.

It is unclear whether the *Babesia* species described in this report is the same species described in raccoons by Birkenheuer et al. (2006). While the genes sequenced in this study suggest that the raccoon and maned wolf isolates are very closely related, the sequences of single genes alone cannot define a species (do Nascimento et al., 2012). Similarly, whole-genome sequencing of *Babesia* isolates from raccoons and maned wolves may need to be performed to understand the taxonomy of these organisms.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ttbdis.2018.09.005.

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