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Detection of a *Theileria* species in dogs in South Africa

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Abstract

A *Theileria* species was detected by PCR in blood samples collected from dogs in the Pietermaritzburg area and was also found in dogs presented at the Outpatients Clinic of the Onderstepoort Veterinary Academic Hospital (OVAH), in the Pretoria area, South Africa. In the Pietermaritzburg area, 79 of the 192 samples were positive, while 3 out of 1137 of the Onderstepoort samples were positive. Three positive samples from Pietermaritzburg were co-infected with *Ehrlichia canis*. PCR positive samples were further analysed by the Reverse Line Blot (RLB) and sequence analysis. Phylogenetic analysis of the 18S rRNA full-length gene sequences of one sample (VT12) from Pietermaritzburg and two samples from OVAH (BC281 and BC295) revealed a close relationship with sequences of *Theileria* species (sable). Clinical signs of the dogs that were examined at Pietermaritzburg and OVAH included an immune-mediated condition with severe thrombocytopenia. These findings identify a *Theileria* sp. in dogs for the first time in South Africa and add yet another microorganism to the growing list of haemoprotozoan parasites infecting dogs worldwide. The clinical significance of this infection in dogs is poorly resolved.

Keywords: *Theileria* sp.; Dogs; Thrombocytopenia; South Africa

1. Introduction

Canine babesiosis, a haemolytic disease of significant economic importance, is the most frequently encountered tick-borne protozoal infection of dogs in South Africa (Shakespeare, 1995; Collett, 2000). The parasites associated with canine babesiosis in South Africa are *Babesia rossi* and *Babesia vogeli* (Matjila et al., 2004). *B. rossi*, which causes a severe disease that can be life-threatening, is the most prevalent species isolated from dogs presented at the Onderstepoort Veterinary Academic Hospital (OVAH) (Böhm et al., 2006). The clinical signs and pathology of the disease may include pyrexia, splenomegaly, anaemia, haemolysis and haemoglobinuria, icterus, circulatory collapse, multiple organ failure and neurological signs (Jacobson and Clark, 1994). The clinical signs of infection caused by *B. vogeli* infection has not been well documented in South Africa (Böhm et al., 2006), although *B. vogeli* has been detected in dogs diagnosed with clinical babesiosis presented at the Outpatients Clinic, OVAH (Böhm et al., 2006). Elsewhere *B. vogeli* infections have been reported to cause only a mild disease in dogs (Uilenberg et al., 1989).

Recent publications have reported on previously unknown pathogens that infect dogs and cause a

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haemolytic syndrome. Firstly, a novel large *Babesia* sp. has been identified in dogs in North America (Birkenheuer et al., 2004). The parasite identified was isolated from the bone marrow as well as the blood of a dog with haematological abnormalities consistent with babesiosis (Birkenheuer et al., 2004). Secondly, *Rangelia vitally*, a blood parasite causing a disease characterized by anaemia, jaundice, fever, splenomegaly, lymphadenopathy, haemorrhage in the gastrointestinal tract and persistent bleeding from the nose, has been described in Brazil (Loretti and Barros, 2005). *R. vitally* is suspected to be tick-transmitted and the authors have stated that the parasite is a protozoan of the phylum Apicomplexa, although different from *Babesia*, since it has an intra-endothelial stage. These authors did not report on any molecular comparisons, which limited determination of the phylogenetic relationship to other blood protozoan parasites. Thirdly, small babesias with similar morphology to *B. gibsoni* have also been described (Kjemtrup et al., 2000, 2006). Although similar in morphology to *B. gibsoni*, these parasites are genetically distinct and include an Asian isolate, a Spanish isolate and a Californian isolate (Kjemtrup et al., 2000). Recent molecular research has shown that the Californian isolate is genotypically and phenotypically different from the *B. gibsoni* group, and has thus been named *Babesia conradae* (Kjemtrup et al., 2006).

There have been no reports of pathogenic *Theileria* species in dogs. The only species associated with a haemolytic disease of dogs is the *Babesia microti*-like, controversially named parasite, *Theileria annae* (Zahler et al., 2000; Camacho et al., 2001, 2004; Camacho Garcia, 2006). Morphologically, this parasite has been described as a small piroplasm (Camacho Garcia, 2006). Molecular analysis of the 18S rRNA gene has shown that this parasite is closely related to *B. microti*, a rodent parasite (Zahler et al., 2000; Criado-Fornelio et al., 2003a; Conrad et al., 2006). Several authors have cited Goethert and Telford (2003) when referring to this parasite as *Babesia annae*. Although Goethert and Telford (2003) did not propose the name *B. annae*, they questioned the use of *Theileria* as a genus name since no evidence was presented by Zahler et al. (2000) for a pre-erythrocytic or lymphocyte-infecting stage, nor was there any evidence for the absence of transovarial transmission in ticks (Goethert and Telford, 2003). The provisional assignment to the genus *Theileria* reflects a controversial argument by some parasitologists working with piroplasms that the small *Babesia* should be removed from the genus *Babesia* (Guitián et al., 2003). In the context of our report we will, therefore, refer to the said parasite as *T. annae*.

Other *Theileria* sp. that have been reported from dogs are *Theileria annulata* (Criado et al., 2006) and *Theileria equi* (Criado-Fornelio et al., 2003b). *Theileria annulata* was detected from an asymptomatic dog (Criado et al., 2006), whereas *T. equi* was detected from three asymptomatic dogs and one symptomatic dog (Criado-Fornelio et al., 2003c). These findings were followed up, however, and as far as we know none of these *Theileria* parasites have subsequently been isolated from clinically reacting dogs. Our report describes a *Theileria* sp. isolated from dogs originating from two localities in South Africa, namely Pietermaritzburg (KwaZulu-Natal) and the Onderstepoort district of Pretoria (Gauteng). The *Theileria* sp. was first detected from samples collected in Pietermaritzburg in 2004. The DNA of this organism was later also detected in two clinical samples collected from two dogs presented at the OVAH in 2005. The same DNA as this organism was detected in a third clinical sample, collected from a dog presented at the OVAH in January 2007.

2. Materials and methods

Blood samples ($n = 192$) were collected monthly over a six-month period from the Pietermaritzburg area, during the early summer months of 2004, and late summer months of 2005. The samples were collected routinely from dogs involved in a study of tick-repellent impregnated dog collars. Blood samples ($n = 1137$) were collected from dogs presented at the OVAH from January 2002 to January 2007. Blood-smear examinations were done by the attending clinicians on all samples. Blood samples were then collected into EDTA Vacutainer® (Franklin Lakes, USA) tubes and sent to the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria, for molecular analysis.

DNA was extracted from 200 μl of each blood sample using the QIAmp® blood and tissue extraction kit (Qiagen, Hilden, Germany), following the manufacturer's protocols. PCR was performed with primers RLB-F2 (5′-GAC ACA GGG AGG TAG TGA CAA G-3′) and RLB-R2 (biotin-5′-CTA AGA ATT TCA CCT CTG ACA GT-3′) amplifying a fragment of 460–540 bp from the 18S rRNA gene spanning the V4 region (Gubbels et al., 1999; Matjila et al., 2004). The conditions for the PCR included an initial step of 3 min at 37 °C, 10 min at 94 °C, 10 cycles of 94 °C (20 s)–67 °C (30 s)–72 °C (30 s), with lowering of annealing step after every second cycle by 2 °C (touchdown PCR). The reaction was then followed by 40 cycles of

denaturation at 94 °C for 30 s, annealing at 57 °C for 30 s and extension at 72 °C for 30 s. PCR-amplified products were tested with the RLB, as previously described (Matjila et al., 2004). An additional plasmid control was used as an internal positive control to ensure that all *Babesia* species-specific probes were correctly bound to the RLB membrane and that they were functional (Matjila et al., 2005).

PCR products that did not hybridize to any of the species-specific probes but hybridized to the *Theileria* genus-specific probe were selected from the samples collected in Pietermaritzburg and Onderstepoort. The RLB was repeated using a new membrane which included *Theileria* probes described by Nijhof et al. (2005). Samples, VT4, VT9, VT12 and VT17 collected from Pietermaritzburg and samples BC285 and BC295 and BC610 collected from the OVAH were partially sequenced (400–540 bp) using primers RLB F2 and RLB R2. These samples were selected for sequencing based on the quality and quantity of their genomic DNA. A BLAST search was performed with the obtained sequences using the BLASTn algorithm and compared with sequences deposited in GenBank. The full-length 18S rRNA gene of sample VT12 (Pietermaritzburg) and the two clinical samples, BC281 and BC295 (OVAH) were amplified using 20 pmol of primers Nbab 1F (5′-AAG CCA TGC ATG TCT AAG TAT AAG CTT TT-3′) and TB Rev (5′-AAT AAT TCA CCG GAT CAC TCG-3′) to give a PCR amplicon of *ca* 1800 base pairs that was subsequently visualized by gel electrophoresis.

These PCR products were purified with the QIAmp® PCR purification kit (Qiagen, Hilden, Germany), and sent for sequencing at the Genetics Section of the Faculty of Veterinary Science. The full-length 18S rRNA gene was sequenced in parts using 3.2 pmol of the following primers: Nbab1F (5′-AAG CCA TGC ATG TCT AAG TAT AAG CTT TT-3′) (Oosthuizen et al., 2008), TB Rev (5′-AATAATTCACCGGAT-CACTCG-3′), BT 2R (5′-CCC GTG TTG AGT CAA ATT AAG CCG-3′), BT 3F (5′-GGG CAT TCG TAT TTA ACT GTC AGA GG-3′), (Oosthuizen et al., 2008), Nbab 4F (5′-CCG TTA ACG GAA CGA GAC CTT AAC C-3′) and Nbab 4R (5′-GGT AGG CCA ATA CCC TAC CG-3′).

DNA amplicons of sample VT12, BC281 and BC295 were also cloned into the pGem T easy vector (Promega, Leiden, The Netherlands) following the manufacturer's instructions. Twelve clones of each sample containing the amplified product were then sequenced using primers SP6 (5′-TAA ATC CAC TGT GAT ATC TTA TG-3′) and T7 (5′-TAT GCT GAG TGA TAT CCC GCT-3′). Sequence data for the full-length 18S rRNA gene were assembled and edited to a total length of 1627 bp using GAP 4 of the Staden package (Version 1.6.0 for Windows) (Bonfield et al., 1995; Staden, 1996; Staden et al., 2000), and deposited in GenBank. The sequences were aligned with sequences of related genera using ClustalX (Version 1.81 for Windows). The alignment was manually truncated to the size of the smallest sequence (∼1368 bp). The two-parameter model of Kimura and the Jukes and Cantor correction model for multiple base changes were used to construct similarity matrices (Jukes and Cantor, 1969; Kimura, 1980). Neighbor-joining (Saitou and Nei, 1987) and the maximum parsimony methods were used for the construction of phylogenetic trees using the Mega 3.0 software package (Kumar et al., 2004). The methods above were used in combination with the bootstrap method (Felsenstein, 1985)(1000 replicates/tree for distance methods and 100 replicates/tree for parsimony methods).

3. Results

Some of the processed samples were negative on blood-smear examination for piroplasms, but were suspected to be *Babesia* positive. Initial processing of blood samples using the RLB assay revealed that 76 of the 192 blood samples from Pietermaritzburg were positive for a *Theileria* sp. by hybridizing with a *Theileria/Babesia* genus-specific catchall probe as well as the *Theileria* genus-specific catchall probe (Table 1). Three of the 1137 samples collected from the OVAH, were positive for a *Theileria* sp. by also hybridizing with the same *Theileria/Babesia* genus-specific probe

Table 1
Reverse line blot hybridization results of dogs positive for only *Theileria* sp. and for mixed infections of *Theileria* sp. and *E. canis*

Location	Total number of collected samples	Number of samples positive for *Theileria* sp.	Number of samples positive for *Theileria* sp. and *E. canis*
Pietermaritzburg	192	76	3
OVAH	1137	3	–

NB: Samples from both localities were positive for other important blood parasite. The results of these are reported in a separate manuscript.

as well as the *Theileria* genus-specific catchall probe. Selection and partial sequencing (400–500 bp) of samples VT4, 9, 12 and 17 from Pietermaritzburg and samples BC281, 295 and 610 from OVAH revealed that the samples were similar to the previously described *Theileria* sp. characterized from sable antelope (*Hippotragus niger*) (Stoltsz and Dunsterville, 1992). Repeated testing of all samples on the RLB membrane that had species-specific probes that included *Theileria* sp. (greater kudu), *Theileria* sp. (grey duiker), *Theileria* sp. (sable) (Nijhof et al., 2005) and *T. annae* (CCG AAC GTA ATT TTA TTG ATT TG)

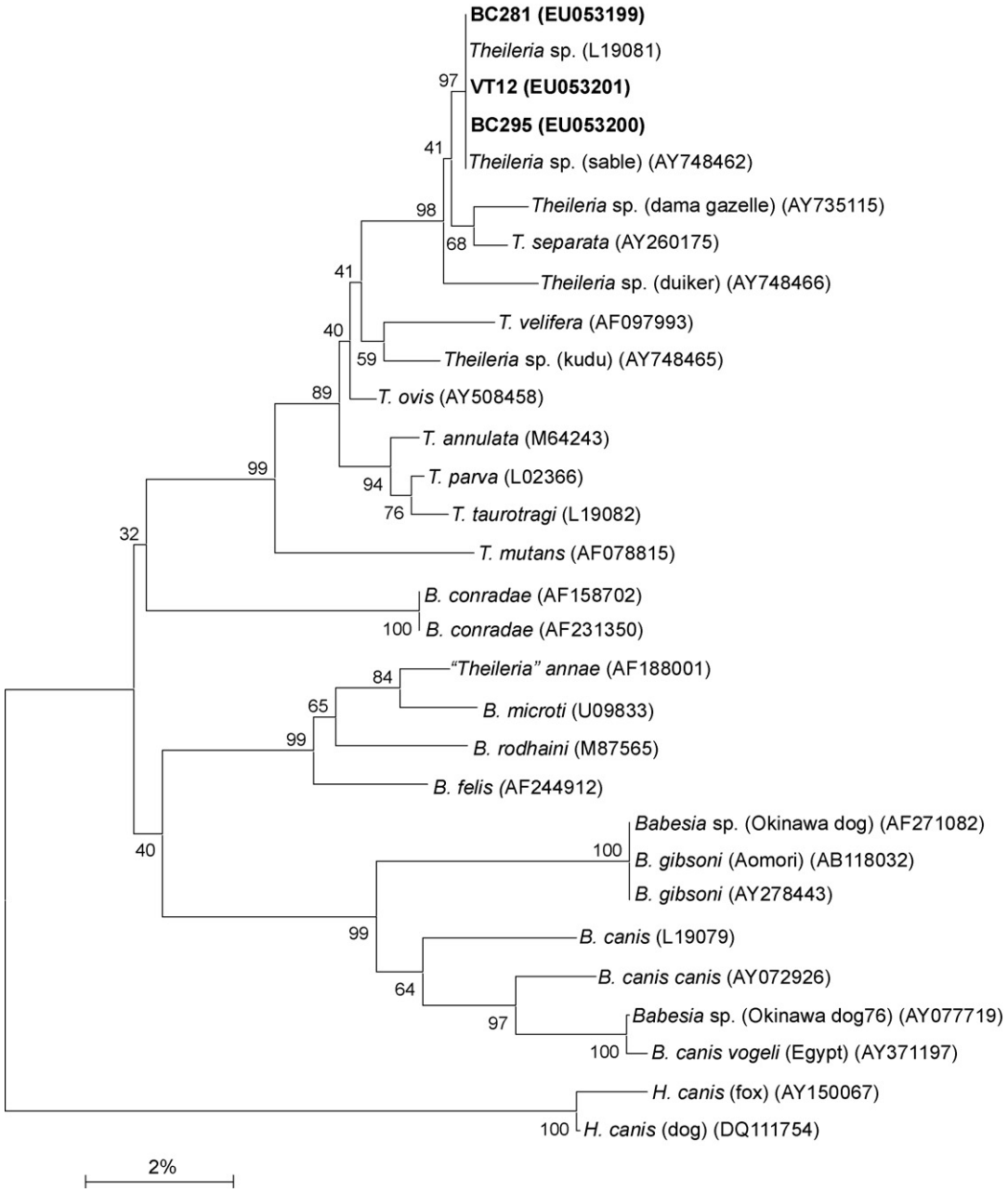

Fig. 1. Neighbor-joining tree, with the Kimura two-parameter distance (Kimura, 1980) calculation showing the phylogenetic relationship of BC281, 295 and VT12 to related species based on the 18S rRNA gene sequences. Relationships are presented as an unrooted tree with branch lengths being proportional to the estimated genetic distance between the strains. The scale bar represents the % nucleotide difference. The GenBank accession numbers are indicated in parentheses.

revealed that all the previously *Theileria* genus-specific positive samples hybridized with the *Theileria* sp. (sable) probe. Three further blood samples from Pietermaritzburg were concurrently infected with *Theileria* sp. and *Ehrlichia canis*, as detected by the RLB. Blood-smear examinations of Pietermaritzburg and OVAH samples did not contain any *Theileria*-infected leukocytes and/or red blood cells, but there were other important haemoparasites (including *B. rossi*, *E. canis* and mixed infections of *B. rossi* and *E. canis*) of dogs detected in blood samples by light microscopy and/or PCR/RLB, collected from Pietermaritzburg and from OVAH (Matjila et al., 2008).

Full-length 18S rRNA gene sequences of samples VT12 (EU053201) from Pietermaritzburg and two samples from OVAH BC281 (EU053199) and BC295 (EU053200) were compared with sequences of related genera. The BLAST search revealed highest similarities (∼99%) with a *Theileria* sp. (AY748462) isolated from a sable antelope originating from Malelane (southern Kruger National Park area of South Africa), and a *Theileria* sp. (L19081) that was also isolated from a sable antelope and later described and named: *Theileria* sp. (sable) (Allsopp et al., 1994). Samples VT12, BC281 and 295 also showed ∼98% similarity with two *Theileria* sp. isolated from Texas (USA) dama gazelle (AY735116 and AY735115) and with *Theileria separata* (AY260175). These similarities were confirmed by both neighbor-joining and maximum parsimony phylogenetic approaches. No significant changes in the topology of the trees, or in the bootstrap values, were found when using any of the phylogenetic analysis procedures. The representative tree obtained by the neighbor-joining method with the Kimura two-parameter distance calculation (Kimura, 1980), is based on a 1368 bp region of the 18S rRNA gene (Fig. 1). In the aligned region, isolates VT12, BC281 and BC295 showed a one bp difference with *Theileria* sp. (sable) (AY748462) and four bp differences and a deletion with *Theileria* sp. (sable) (L19081).

4. Discussion

The only *Theileria* sp. currently known to cause disease in domestic dogs is the *B. microti*-like, *T. annae* (Zahler et al., 2000; Guitián et al., 2003; Camacho et al., 2004; Camacho Garcia, 2006), which has only been reported to occur in Spain. *T. annae* has been reported to cause a disease characterized by apathy, fever, and anaemia (Zahler et al., 2000). Severe regenerative anaemia and thrombocytopenia have been reported to be a constant characteristic of *T. annae* infection (Camacho Garcia, 2006). The level of parasitaemia is also usually low and not statistically related to the severity of the anaemia or renal failure (Camacho Garcia, 2006).

In our study we used molecular techniques to identify a *Theileria* species of dogs associated with a haemolytic disease. No other causes of clinical signs could be identified in the affected dogs. The Pietermaritzburg samples were part of an independent study on acaricide-impregnated dog collars. This made it difficult for us to obtain the exact histories of dogs that tested positive for the *Theileria* sp. and/or *E. canis*. However, from the brief histories of samples that we received from dog samples VT5, 6, 14, 17 and 21, we gathered the following information. (1) The dog yielding sample VT5 had a history of anaemic episodes, which seemed to respond well to steroid treatment. This could be indicative of an immune-mediated disease. (2) Sample VT6 was collected from a 4-year-old dog, which had anorexia, fever, abdominal pain and respiratory difficulty. No piroplasms were seen in smear examination and the dog was suspected to have an immune-mediated condition. (3) Sample VT14 was collected from a dog with abdominal pain and suspected colitis. (4) Sample VT17 was collected from a 5-year-old dog presented with weight loss and fever. Smear examination of VT17 showed suspected *Babesia*-infected erythrocytes and a regenerative anaemia. However, this sample was PCR/RLB negative for *Babesia*. Further details were not provided. Finally (5) sample VT21 was collected from a 2-year-old emaciated dog with heavy hook-worm infection and thrombocytopenia. With the exception of VT14, findings were consistent with canine babesiosis (fever, anorexia, anaemia and thrombocytopenia) or similar to those described in dogs diagnosed with *T. annae* infection (fever, anaemia, and thrombocytopenia).

Detailed clinical histories were obtained from three *Theileria*-positive samples (BC281, 295 and 610) collected at the OVAH. Sample BC281 was collected from a 4-year-old Doberman Pinscher diagnosed with chronic-active necrotic superficial dermatitis and deep cellulitis of unknown cause, anaemia and severe thrombocytopenia. The dog was again seen three months later, when it was diagnosed with nasal trauma and severe thrombocytopenia. PCR/RLB analysis of the blood sample revealed that the dog was infected with *Theileria* sp. No *Ehrlichia* and/or *Anaplasma* infections were detected from sample BC281.

Sample BC295 was collected at the OVAH, from a two-and-a-half-month-old Miniature Schnauzer. On clinical examination the dog had a fever and bloody

diarrhoea. The dog was diagnosed with parvovirus infection, based on clinical signs. PCR/RLB tests confirmed a *Theileria* sp. infection only. A month later, the dog was brought back to the clinic and was diagnosed with distemper and parvovirus, infection based on clinical signs. Blood samples taken on this second occasion again indicated a *Theileria* sp. infection by PCR/RLB tests.

Sample BC610 was collected from a dog admitted for splenomegaly diagnosed at OVAH. Haematology revealed severe thrombocytopenia and abdominal ultrasound demonstrated an enlarged spleen. The dog's condition worsened and an emergency splenectomy was performed. The thrombocyte count returned to normal the following day. It was thus suspected that the thrombocytopenia was as a result of sequestration or immune-mediated destruction of thrombocytes. PCR/RLB tests confirmed a *Theileria* sp. infection and no *Ehrlichia* and/or *Anaplasma* infection. Smear examinations of the three OVAH samples (BC281, 295 and 610) did not show any piroplasms, but may have been under the detection limits for routine light microscopy as often encountered in *T. annae* infections (Camacho Garcia, 2006).

Although the pathophysiology of the detected *Theileria* sp. in dogs is unknown, it is apparent from the few cases described here that anaemia (possibly haemolytic), splenomegaly and a possible immune-mediated syndrome may be associated with this organism. Similar clinical signs are normally seen in dogs infected with *T. annae* (Camacho Garcia, 2006) including haematological disorders such as thrombocytopenia, which is a common finding in the absence of *Ehrlichia* infection in 75% of dogs infected with *T. annae* (Camacho Garcia, 2006). Phylogenetic analysis (Fig. 1) of the *Theileria* sp. in dogs characterized in this study (BC281: Accession number: EUO53199; BC295: Accession number: EUO53200; and VT12: Accession number: EUO53201) showed a close similarity, with one base pair difference only, to *Theileria* sp. (sable) (AY748462), four base differences to *Theileria* sp. (sable) (L19081) and no phylogenetic relationship to *T. annae* (AF188001). Both *Theileria* (sable) species cause mortalities in sable antelopes (Nijhof et al., 2005). To our knowledge none of the dogs that the *Theileria* sp. was isolated from died as a result of the infection. As previously suggested, this may indicate evidence of a chronic established host–parasite relationship (Ebert, 1998), or it may indicate that the *Theileria* sp. (dog) is not as virulent in dogs as *Theileria* sp. (sable) is in sable antelopes (Nijhof et al., 2005). It has been shown that parasites that are known to be virulent in their typical hosts may infect incidental host without causing disease (Criado-Fornelio et al., 2003c). We can therefore currently only speculate on the clinical relevance of the detected *Theileria* sp. in our sampled dogs. Our findings identify a *Theileria* sp. in dogs for the first time in South Africa and add yet another microorganism to the list of haemoprotozoans infecting dogs. More clinical samples and data will need to be collected and analysed to understand the importance of the *Theileria* sp. We will therefore refer to this parasite as "*Theileria* sp. (dog)" which we found in South Africa.

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