Chapter 6: Detection of *Theileria* sp. infections in dogs in South Africa.

6.1. Abstract

A *Theileria* sp. was detected by PCR in blood samples collected from dogs in the Pietermaritzburg area and 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 79/192 samples were positive, while 3/527 of the Onderstepoort samples were positive. Three positive samples from Pietermaritzburg were co-infected with *Ehrlichia canis*. Parasite DNA was detected and identified by PCR and Reverse Line Blot (RLB), followed by sequence analysis. Phylogenetic analysis of the 18S rRNA full-length sequences of one sample from Pietermaritzburg (VT12: Accession number EU053201) and two samples from OVAH (BC281, Accession number: EU053199 and BC295, Accession number: EU053200) revealed that the isolates were closely related to the published isolates of *Theileria* sp. (sable) (AY74862) and *Theileria* sp. (sable) (L19081). Clinical signs of the dogs that were examined at OVAH and from which the three blood samples were collected were not consistent with theileriosis. All dogs had a similar 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. The clinical significance of this infection in dogs is poorly resolved.

6.2. 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, Penzhorn, Bekker, Nijhof and Jongejan, 2004). *Babesia rossi* which causes a severe disease that can be life-threatening, is the most prevalent species isolated from dogs presented at Onderstepoort Veterinary Academic Hospital (OVAH) (Böhm, Leisewitz, Thompson and Schoeman, 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 (Bohm 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, Franssen, Perie and Spanjer, 1989).

Recent publications have reported on previously unknown pathogens that infect dogs and cause a haemolytic syndrome. A novel large *Babesia* sp. has been identified in dogs in North America (Birkenheuer, Neel, Ruslander, Levy and Breitschwerdt, 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). *Rangelia vitalli*, 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). *Rangelia vitalli* 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. The authors did not report on any molecular comparisons, which limited determination of the phylogenetic relationship to other blood protozoan parasites. Small babesias with similar morphology to *B. gibsoni* (*senso lato*) have been described lately (Kjemtrup, Kocan, Whitworth, Meinkoth, Birkenheuer, Cummings, Boudreaux, Stockham, Irizarry-Rovira and Conrad, 2000; Kjemtrup, Wainwright, Miller, Penzhorn and Carreno, 2006). Although similar in morphology to *B. gibsoni* (*senso lato*), the 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 the Californian isolate to be genotypically and phenotypically different from the *B. gibsoni* (*senso lato*) group. It has thus been named *Babesia conradae* (Kjemtrup et al., 2006).

Until recently there had not been any reports on pathogenic *Theileria* sp. in dogs. *Theileria annae* was the first *Theileria* species to be associated with a haemolytic disease of dogs (Zahler Rinder, H., Schein, E., Gothe, 2000; Camacho, Pallas, Gestal, Guitian, Olmeda, Goethert and Telford, 2001; Camacho, Guitian, Pallas, Gestal, Olmeda, Goethert, Telford and Spielman, 2004) . A number of authors cited Goethert and Telford (2003) when referring to this parasite as *Babesia annae*. Goethert and Telford (2003) did not propose the name *B. annae*, however, but disputed 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). Current molecular evidence based on the analysis of the 18S rRNA gene justifies the naming of *T. annae* for this parasite (instead of *B. annae*) and it will be referred to as such in our report.

Other *Theileria* sp. that have been detected in dogs are *Theileria annulata* (Criado Martinez, Buling, Barba, Merino, Jefferies and Irwin, 2006) and *Theileria equi* (Criado-Fornelio Martinez-Marcos, Buling-Sarana and Barba-Carretero, 2003a). *Theileria annulata* was detected from an asymptomatic dog (Criado et al., 2006) whereas *Theileria equi* was detected from three asymptomatic dogs and one symptomatic dog (Criado-Fornelio et al., 2003a). 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. This 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 detected in two clinical samples collected from two dogs presented at the Outpatients Clinic of the OVAH in 2005. The same DNA found in this organism was detected in a third clinical sample, collected from a dog presented at the Outpatients Clinic in January 2007.

6.3. Materials and Methods

6.3.1. Collection of samples

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=527) were collected from dogs in need of veterinary care presented at the Outpatients Clinic, OVAH from January 2002 to January 2007. Blood-smear examinations were done by the attending clinicians on all samples. Blood smear examinations did not always reveal piroplasms. 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 detection of parasites.

6.3.2. DNA extraction

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.

6.3.3. PCR

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 460-540bp from the 18S rRNA gene spanning the V4 region (Gubbels, de Vos, Van der Weide, Viseras, Schouls, de Vries and Jongejan, 1999; Matjila et al., 2004). The conditions for the PCR included an initial step of 3 min at 37º C, 10 min at 94ºC, 10

cycles of 94ºC (20s)- 67 ºC (30s)- 72º C (30s), with lowering of annealing step after every second cycle by 2º C (touchdown PCR). The reaction was then followed by 40 cycles of denaturation at 94º C for 30s, annealing at 57º C for 30s and extension at 72º C for 30s.

6.3.4. Reverse line blot hybridisation

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, Nijhof, Taoufik, Houwers, Teske, Penzhorn, de Lange and Jongejan, 2005).

6.3.5. Sequencing

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, Pillay, Steyl, Prozesky, Stoltsz, Lawrence, Penzhorn and Jongejan (2005). Samples, VT4, VT9, VT12 and VT17 collected from Pietermaritzburg and samples BC285 and BC295 and BC610 collected from the OVAH were partially sequenced (400-540bp) 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, Zweygarth Collins, Troskie and Penzhorn, 2008), TB Rev (5'-AAT AAT TCA CCG GAT CAC TCG-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').

6.3.6. Phylogenetic analysis

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, Smith and Staden, 1995; Staden, 1996; Staden, Beal and Bonfield, 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). As only partial sequences (415bp) of *T. annae* were available in Genbank (AY150068 and AY150069), *T. annae* was omitted from further phylogenetic analysis. 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, Tamura and Nei, 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).

6.4. 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 (Table 6.1) 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.

Three of the 527 samples collected from the Outpatients Clinic, OVAH, were positive for a *Theileria* sp. by also hybridizing with the same *Theileria*/*Babesia* genus-specific probe as well as the *Theileria* genus-specific catchall probe. Selection and partial sequencing (400-500bp) 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 the 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 *Theileria annae* (CCG AAC GTA ATT TTA TTG ATT TG) 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 show 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 PCR/RLB, collected from Pietermaritzburg and from OVAH (Matjila, Leisewitz, Jongejan and Penzhorn, 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, Cavalier-Smith, De Waal, and Allsopp, 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. 6.1). 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).

6.5. Discussion

The only *Theileria* sp. currently known to cause disease in domestic dogs is the *B-microti*-like, *T. annae* (Zahler et al., 2000; Camacho et al., 2004; Garcia, 2006), which has only been reported to occur in Spain. *Theileria equi* and *T. annulata* have also been isolated from dogs in Spain (Criado et al., 2006; Criado-Fornelio et al., 2003a). *Theileria 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 (Garcia, 2006). The level of

parasitaemia is also usually low and not statistically related to the severity of the anaemia or renal failure (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 private group's commercial study on acaricide-impregnated dog collars used as a prophylactic measure against canine babesiosis. 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. The dog yielding sample VT5 had a history of anaemic episodes, which seemed to respond well to steroid treatment. This could be indicative of any inflammatory or an immune-mediated disease. At the time of collecting sample VT6 (from a 4-year-old dog), the dog had a depressed habitus, anorexia, fever, abdominal pain and respiratory difficulty. No piroplasms were seen during smear examination and the dog was suspected to have an immune-mediated condition. Sample VT14 was collected from a dog with abdominal pain and suspected colitis, whereas sample VT17 was collected from a five-year-old German Shepherd dog presented with weight loss and a 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 two-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 the three *Theileria*-positive samples (BC281, 295 and 610) collected at the Outpatients Clinic (OVAH). Sample BC281 was collected from a four-year-old Doberman Pinscher diagnosed with chronic-active necrotic superficial dermatitis and deep cellulitis of uncertain cause, anaemia and severe thrombocytopenia. Diagnostic ultrasound of the abdomen indicated that there was a mild iliac lymph node enlargement. 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 a *Theileria* sp. No *Ehrlichia* and / or *Anaplasma* infections were detected from sample BC281.

Sample BC295 was collected at the Outpatients Clinic, 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 Outpatients 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 the Outpatient Clinic, 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 piroplasm infections. It should be borne in mind, however, that the parasite density may have been below the level of detection for routine light microscopy as often encountered in *T. annae* infections (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* (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* (Garcia 2006). Phylogenetic analysis (Fig. 6.1) 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 to *Theileria* sp. (sable) (AY748462) and four base differences to *Theileria* sp. (sable) (L19081). 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 parasite is not as virulent in dogs as it 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, Martinez-Marcos, Buling-Sarana and Barba-Carretero, 2003b).

6.6. Conclusion

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.

6.7. Figures and Tables

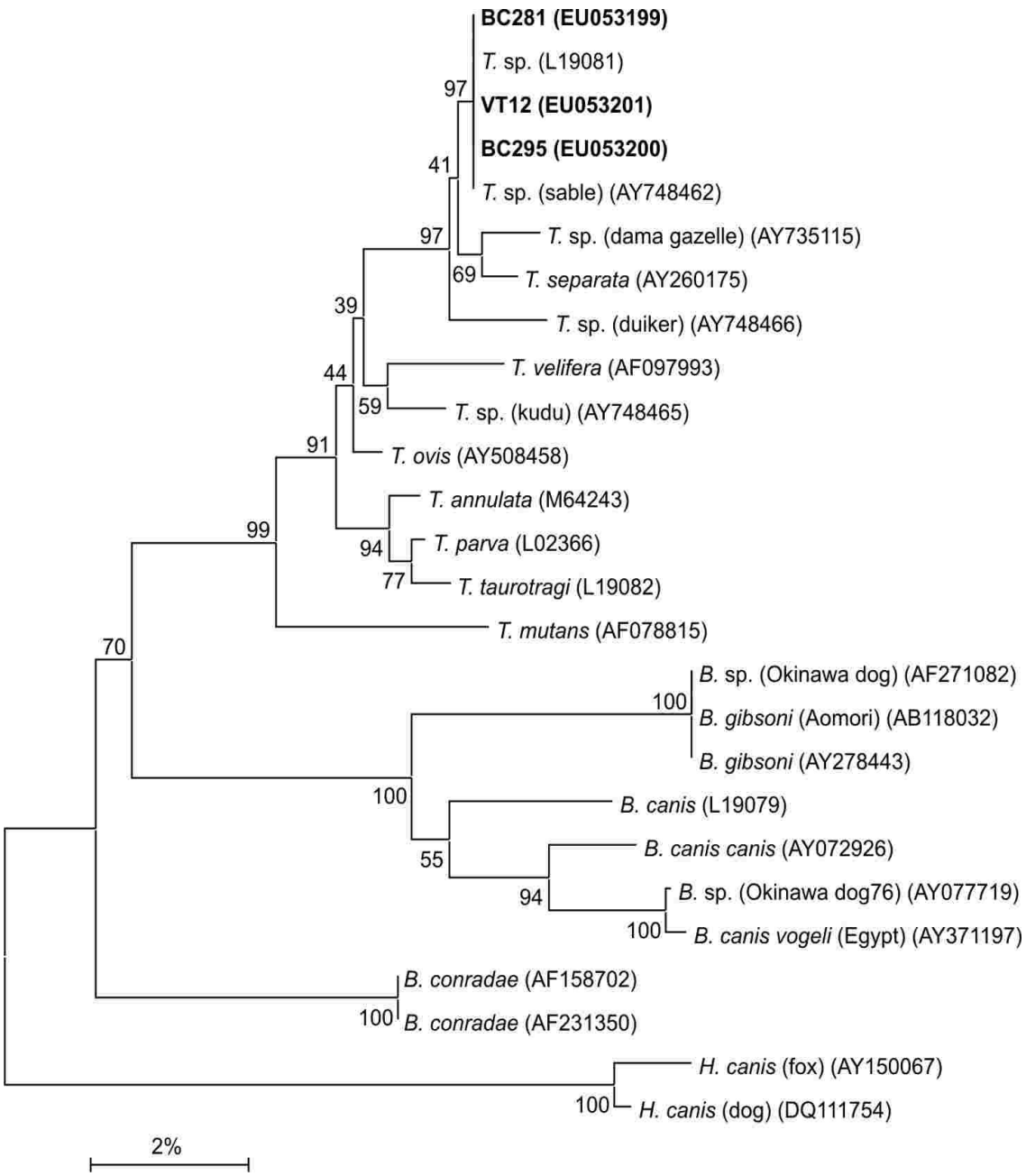

Figure 6.1: Neighbor-joining tree, with the Kimura two-parameter distance (Kimura, 1980) calculation showing the phylogenetic relationship of BC281, 295 & VT12 to related species based on the 18S rRNA gene sequences. Relationships are presented as an unrooted tree with branch lengths being proportional to the estimated genetic distance between the strains. The scale bar represents the % nucleotide difference. The GenBank accession numbers are indicated in parentheses.

Table 6.1: 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	527	3	-

NB: Samples from both localities were positive for other important blood parasite. The results of these are reported in chapter 3

6.8. References

Allsopp, M.T., Cavalier-Smith, T., De Waal, D.T., Allsopp, B.A., 1994. Phylogeny and evolution of the piroplasms. Parasitology 108, 147-152.

Birkenheuer, A.J., Neel, J., Ruslander, D., Levy, M.G., Breitschwerdt, E.B., 2004. Detection and molecular characterization of a novel large *Babesia* species in a dog. Veterinary Parasitology 124, 151-160.

Böhm, M., Leisewitz, A.L., Thompson, P.N., Schoeman, J.P., 2006. Capillary and venous *Babesia canis rossi* parasitaemias and their association with outcome of infection and circulatory compromise. Veterinary Parasitology 141, 18-29.

Bonfield, J.K., Smith, K., Staden, R., 1995. A new DNA sequence assembly program. Nucleic Acids Research 23, 4992-4999.

Camacho, A.T., Guitian, E.J., Pallas, E., Gestal, J.J., Olmeda, A.S., Goethert, H.K., Telford, S.R., III, Spielman, A., 2004. Azotemia and mortality among *Babesia microti*-like infected dogs. Journal of Veterinary Internal Medicine 18, 141-146.

Camacho, A.T., Pallas, E., Gestal, J.J., Guitian, F.J., Olmeda, A.S., Goethert, H.K., Telford, S.R., 2001. Infection of dogs in north-west Spain with a *Babesia microti*-like agent. Veterinary Record 149, 552-555.

Collett, M.G., 2000. Survey of canine babesiosis in South Africa. Journal of the South African Veterinary Association 71, 180-186.

Criado, A., Martinez, J., Buling, A., Barba, J.C., Merino, S., Jefferies, R., Irwin, P.J., 2006. New data on epizootiology and genetics of piroplasms based on sequences of small ribosomal subunit and cytochrome b genes. Veterinary Parasitology 142, 238-247.

Criado-Fornelio, A., Martinez-Marcos, A., Buling-Sarana, A., Barba-Carretero, J.C., 2003a. Molecular studies on *Babesia*, *Theileria* and *Hepatozoon* in southern Europe. Part I. Epizootiological aspects. Veterinary Parasitology 113, 189-201.

Criado-Fornelio, A., Martinez-Marcos, A., Buling-Sarana, A., Barba-Carretero, J.C., 2003b. Presence of *Mycoplasma haemofelis*, *Mycoplasma haemominutum* and piroplasmids in cats from southern Europe: a molecular study. Veterinary Microbiology 93, 307-317.

Ebert, D., 1998. Experimental evolution of parasites. Science (Washington) 282, 1432-1435.

Felsenstein, J., 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39, 783-791.

Garcia, A.T.C., 2006. Piroplasma infection in dogs in northern Spain. Veterinary Parasitology 138, 97-102.

Goethert, H.K., Telford, S.R.I., 2003. What is *Babesia microti*? Parasitology 127, 301-309.

Gubbels, J.M., de Vos, A.P., Van der Weide, M., Viseras, J., Schouls, L.M., de Vries, E., Jongejan, F., 1999. Simultaneous detection of bovine *Theileria* and *Babesia* species by reverse line blot hybridization. Journal of Clinical Microbiology 37, 1782-1789.

Jacobson, L.S., Clark, I.A., 1994. The pathophysiology of canine babesiosis: new approaches to an old puzzle. Journal of the South African Veterinary Association 65, 134-145.

Jukes, T.H., Cantor, C.R., 1969. Evolution of protein molecules. In: Munro, H.N. (Ed.), Mammalian Protein Metabolism. Academic Press, New York, pp. 21-132.

Kimura, M., 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. Journal of Molecular Evolution 16, 111-120.

Kjemtrup, A.M., Kocan, A.A., Whitworth, L., Meinkoth, J., Birkenheuer, A.J., Cummings, J., Boudreaux, M.K., Stockham, S.L., Irizarry-Rovira, A., Conrad, P.A., 2000. There are at least three genetically distinct small piroplasms from dogs. International Journal of Parasitology 30, 1501-1505.

Kjemtrup, A.M., Wainwright, K., Miller, M., Penzhorn, B.L., Carreno, R.A., 2006. *Babesia conradae*, sp. nov., a small canine *Babesia* identified in California. Veterinary Parasitology 138, 103-111.

Kumar S, Tamura K, Nei M, 2004. MEGA3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief Bioinformatics 5, 150-163.

Loretti, A.P., Barros, S.S., 2005. Hemorrhagic disease in dogs infected with an unclassified intraendothelial piroplasm in southern Brazil. Veterinary Parasitology 134, 193-213.

Matjila, P.T., Leisewitz, A.L., Jongejan, F., Penzhorn, B.L, 2008. Molecular detection of tick-borne protozoal and ehrlichial infections in domestic dogs in South Africa. Veterinary Parasitology 155, 152-157.

Matjila, P.T., Penzhorn, B.L., Bekker, C.P., Nijhof, A.M., Jongejan, F., 2004. Confirmation of occurrence of *Babesia canis vogeli* in domestic dogs in South Africa. Veterinary Parasitology 122, 119-125.

Matjila, T.P., Nijhof, A.M., Taoufik, A., Houwers, D., Teske, E., Penzhorn, B.L., De Lange, T.D., Jongejan, F., 2005. Autochthonous canine babesiosis in The Netherlands. Veterinary Parasitology 131, 23-29.

Nijhof, A.M., Pillay, V., Steyl, J., Prozesky, L., Stoltsz, W.H., Lawrence, J.A., Penzhorn, B.L., Jongejan, F., 2005. Molecular characterization of *Theileria* species associated with mortality in four species of African antelopes. Journal of Clinical Microbiology 43, 5907-11.

Oosthuizen, M.C., Zweygarth, E., Collins, N.E., Troskie, M., Penzhorn, B.L., 2008. Identification of a novel *Babesia* sp. from sable antelope (*Hippotragus niger*, Harris 1838). Journal of Clinical Microbiology 46, 2247-2251.

Saitou, N., Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Molecular and Biological Evolution 4, 406-425.

Shakespeare, A.S., 1995. The incidence of canine babesiosis amongst sick dogs presented to the Onderstepoort Veterinary Academic Hospital. Journal of the South African Veterinary Association 66, 247-250.

Staden, R., 1996. The Staden sequence analysis package. Molecular Biotechnology 5, 233-241.

Staden, R., Beal, K.F., Bonfield, J.K., 2000. The Staden package, 1998. Methods in Molecular Biology 132, 115-130.

Stoltsz, W.H., Dunsterville, M.T., 1992. In vitro establishment and cultivation of a *Cytauxzoon* sp. (*Theileria* sp.) from a sable antelope (*Hippotragus niger*, Harris 1838). Journal of the South African Veterinary Association 63, 182 (Abstract).

Uilenberg, G., Franssen, F.F., Perie, N.M., Spanjer, A.A., 1989. Three groups of *Babesia canis* distinguished and a proposal for nomenclature. Veterinary Quarterly. 11, 33-40.

Zahler, M., Rinder, H., Schein, E., Gothe, R., 2000. Detection of a new pathogenic *Babesia microti*-like species in dogs. Veterinary Parasitology 89, 241-248.