# Occurrence of blood-borne tick-transmitted parasites in common tsessebe (*Damaliscus lunatus*) antelope in Northern Cape Province, South Africa

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**Abstract**

Blood samples were collected from 71 tsessebes relocated from the deproclaimed Vaalbos National Park to Mokala National Park, South Africa. DNA was extracted from the samples and the reverse line blot (RLB) hybridization technique was used to detect and identify any haemoparasites present. Six samples hybridized to the *Theileria/Babesia* genus-specific probe, the *Theileria* genus-specific probe and the *Theileria* sp. (sable) probe, while 3/6 also hybridized to the *Theileria separata* probe. Full-length 18S rRNA genes of the *Theileria* spp. detected were amplified, cloned and sequenced. Two novel *Theileria* 18S rRNA gene sequences were identified which are phylogenetically very closely related to both *Theileria* sp. (sable) and *T. separata*. All animals appeared to be in good health. It seems likely, therefore, that these *Theileria* spp. do not cause disease under normal circumstances. Nevertheless, care should be taken when translocating wild animals, as introduction of novel piroplasm parasites into new areas could cause clinical disease and losses in naïve wildlife and domestic animals, and new parasite species could become established in areas in which they previously did not occur.

## 1. Introduction

The tsessebe (*Damaliscus lunatus*) is listed as "endangered" (Red Data Book, CBS Group, EWT & IUCN, 2004 [Electronic resource]). Decline in tsessebe populations has been ascribed to environmental factors, e.g. dry-season rainfall and competition for grazing with cattle (Dunham et al., 2003, 2004). The decrease in numbers is cause for concern and reserves are developing strategies in an attempt to explain this phenomenon, and cope with it, notably in the Kruger National Park (Grant and van der Walt, 2000). Although *Theileria*-like piroplasms have been reported from tsessebe in Botswana (Carmichael and Hobday, 1975) and fatal cytauxzoonosis has been reported in a tsessebe calf (Jardine, 1992), the role of piroplasms as a possible cause of morbidity and mortality in tsessebe has not been investigated.

When tsessebe were relocated from the deproclaimed Vaalbos National Park (VNP) to Mokala National Park, South Africa, the population was sampled to determine prevalence of piroplasm infections. A reverse line blot (RLB) assay using species-specific oligonucleotide probes based on species-specific sequences in piroplasm 18S rRNA genes (Gubbels et al., 1999), previously used to identify piroplasms in various wildlife species (Nijhof et al., 2003, 2005, Oosthuizen et al., 2008, 2009), was employed.

## 2. Materials and methods

The tsessebe population (n = 215) in VNP was caught by conventional plastic "boma" mass-capture technique, which involves chasing animals into a funnel shaped enclosure using a helicopter, in July and September 2006. Blood was collected from the jugular vein into "Vacutainer" tubes (EDTA) from 71 tsessebe (n=27 in July, n=44 in September), representing one third of the total population. Animals sampled included a mix of ages and sex, but these data were not recorded. The samples were kept refrigerated until transported to the laboratory.

Ticks were collected opportunistically from 12 tsessebe, special attention being given to the ears and perianal region. The ticks were stored in ethanol and were identified using a dissection microscope.

DNA was extracted from 200 µl of EDTA-anticoagulated blood using a DNA extraction kit (DNeasy Tissue kit, QIAGEN, Southern Cross Biotechnologies, SA), following the manufacturer's protocol, except that DNA was eluted in 100 µl elution buffer AE. DNA samples were stored at -20 ºC.

Primers RLB-F2 (5'-GAC ACA GGG AGG TAG TGA CAA G-3') and RLB-R2 (5'-biotin-CTA AGA ATT TCA CCT CTG ACA GT-3'), specific for *Theileria* and *Babesia* species (Nijhof et al., 2003), and primers E HR-F (5'-GGA ATT CAG AGT TGG ATC MTG GYT CAG-3') and E HR-R (5'-biotin-CGG GAT CCC GAG TTT GCC GGG ACT TYT TCT-3'), specific for *Anaplasma* and *Ehrlichia* species (Bekker et al., 2002), were used to amplify the V4 or V1 hypervariable region of the

parasite small subunit rRNA genes using the PCR reaction conditions and the touchdown thermal cycling programme described by Nijhof et al. (2005). PCR amplicons were analyzed using the RLB hybridization assay (Nijhof et al., 2005) using the *Anaplasma*, *Ehrlichia*, *Theileria* and *Babesia* genus- and species-specific probes listed in Table 1.

Two samples positive for *Theileria* sp. (sable) and *Theileria separata* (samples TS22 and TS23) were selected for further characterization. A 1,600 bp fragment of the 18S rRNA gene was amplified using primers Nbab_1F (5'-AAG CCA TGC ATG TCT AAG TAT AAG CTT TT-3') and Nbab_1R (5'-CTT CTC CTT CCT TTA AGT GAT AAG GTT CAC-3') as described by Oosthuizen et al. (2008). PCR products were purified using the QIAquick PCR purification kit (QIAGEN and Southern Cross Biotechnologies, SA).

Purified PCR products were sent to Inqaba Biotechnical Industries [Pty] Ltd (http://www.inqababiotec.co.za/new/index.html), where they were cloned into the pJET1.2/blunt Cloning Vector (Fermentas, Ontario, Canada). Recombinant plasmids were sequenced using the vector primers pJET1.2 forward (5'-CGA CTC ACT ATA GGG AGA GCG GC-3') and pJET1.2 reverse (5'-AAG AAC ATC GAT TTT CCA TGG CAG-3') primers, as well as the RLB-F2 and RLB-R2 amplification primers. Sequencing was performed at Inqaba Biotechnical Industries [Pty] Ltd. Sequences were assembled and edited using GAP4 of the Staden package (version 1.6.0 for Windows) (Staden et al., 2000) and have been deposited in GenBank under accession numbers HQ179765 and HQ179766.

BLASTN (Altschul et al., 1990) was used to search the public sequence databases for homologous sequences. The assembled sequences were aligned with published sequences of related genera using CLUSTAL W (Thompson et al., 1994) and the alignment was manually truncated to the size of the smallest sequence (1,543 bp). Similarity matrices were constructed from the aligned sequence data by single distance, using the two-parameter model of Kimura (1980). The Jukes and Cantor (1969) correction model was applied for multiple base changes. Phylogenetic trees were constructed using MEGA 3.0 (Kumar et al., 2004) using both neighbor-joining (Saitou and Nei, 1987) and maximum-parsimony. Bootstrapping was applied using 1,000 replicates/tree for the distance method and 100 replicates/tree for the parsimony method (Felsenstein, 1985). Modeltest v.3.7 (Posada and Crandall, 1998) was used to select a TrN+I+G substitution model. Phylogenetic analyses using distance and maximum-likelihood methods were carried out using PAUP* v4b10 (Swofford, 2003). Bayesian analysis was performed using MrBayes v.3.1.2 (Ronquist and Huelsenbeck, 2003). Four simultaneous Markov chain Monte Carlo (MCMC) chains were run for 5,000,000 generations, with sampling every 100 generations for a total of 50,000 samples per run. All consensus trees generated were edited using MEGA4 (Tamura et al., 2007).

## 3. Results

Blood was collected from 71/215 tsessebes. RLB hybridization revealed that 6/71 samples were positive for a *Theileria* species, a prevalence of 8.5%. All six samples hybridized to the *Theileria/Babesia* genus-specific probe, the *Theileria* genus-specific probe and the *Theileria* sp. (sable) probe. Three of the six samples

also hybridized to the *Theileria separata* probe.   None of the samples showed *Anaplasma-*, *Ehrlichia-* or *Babesia*-positive signals.

18S rRNA gene sequences were obtained from three clones from one sample (TS22-11, TS22-26 and TS22-27) and from two clones from another sample (TS23-5 and TS23-6).  The sequences from clones TS22-11 and TS22-27 were identical, while the sequence from clone TS22-26 contained 12 nucleotide differences (99% identity to TS22-11 and TS22-27).  The sequence from clone TS23-5 was almost identical to those of TS22-11 and TS22-27, with a single nucleotide difference.  Similarly, the sequence from clone TS23-6 was almost identical to that of TS22-26, containing an additional nucleotide difference from TS22-11 and TS22-27.  Since these sequences were obtained from cloned PCR products, we cannot rule out the possibility of the introduction of sequence artefacts during the PCR, but the sequencing results do reveal the presence of at least two distinct 18S rRNA gene sequence variants in samples TS22 and TS23: the sequence from TS23-6 was designated Tsessebe 1 (accession number HQ179765) and that from TS22-11 was designated Tsessebe 2 (accession number HQ179766).

A BLASTN search of GenBank revealed that these sequences were most closely related to *Theileria* spp. identified in antelope, deer and sheep.  The most closely related sequences to both Tsessebe 1 and Tsessebe 2, with 99% identity, were *Theileria* sp. (sable) (accession number L19081) (Allsopp et al., 1994), and three sequences in dogs (Matjila et al., 2008), namely *Theileria* sp. VT12 (accession number EU053201), *Theileria* sp. BC295 (accession number EU053200) and *Theileria* sp. BC281 (accession number EU053199).  Other closely related sequences,

with 98% identity, included *Theileria separata* (accession number AY260175), as well as sequences identified in dama gazelle in Texas (accession numbers AY735116 and AY735115).

Alignment of the two new sequences revealed 8 nucleotide differences between Tsessebe 1 and *Theileria* sp. (sable), and 15 nucleotide differences between Tsessebe 2 and *Theileria* sp. (sable). The Tsessebe 1 sequence differed from *T. separata* at 26 positions, while there were 21 nucleotide differences between *T. separata* and Tsessebe 2. Tsessebe 1 was identical to *Theileria* sp. (sable) in the target region of the *Theileria* sp. (sable) RLB probe, while there was one difference between Tsessebe 2 and *Theileria* sp. (sable) in this region. Tsessebe 2 was identical to *T. separata* in the target region of the *T. separata* RLB probe, while there were two differences between Tsessebe 1 and *T. separata* in this region. Therefore, it is almost certain that the RLB detected the Tsessebe 1 gene sequence in all six *Theileria*/*Babesia* genus-positive samples and the Tsessebe 2 sequence in three of them.

Phylogenetic analyses using the neighbor-joining, maximum-likelihood, maximum-parsimony and Bayesian inference methods, all yielded trees with no significant differences in their topologies and high bootstrap or nodal support values. A representative distance tree inferred from the 18S rRNA data is shown in Fig. 1. The sequences obtained in this study fall into a clade containing *Theileria* sp. (sable), *Theileria* sp. VT12, *Theileria* sp. BC295, *Theileria* sp. BC281 and *T. separata*, as well as *Theileria* spp. identified in dama gazelle and grey duiker (*Sylvicapra grimmia*).

All ticks collected were identified as *Rhipicephalus evertsi evertsi*; larvae and nymphs were collected from 9/12 animals, the mean infestations being 16.8 larvae and 7.5 nymphs per host. Six adult ticks were collected, from three tsessebe.

**4. Discussion**

Initial RLB results suggested the presence of *Theileria* sp. (sable) and *T. separata* in some tsessebe blood samples. *Theileria* sp. (sable) has been reported in fatal cases of theileriosis in roan and sable antelope (Nijhof et al., 2005), but there is no previous record of *T. separata* infecting antelope species. *T. separata*, which causes subclinical infections in sheep, is transmitted by *R. evertsi evertsi* and is endemic in southern Africa (Lawrence et al., 1994; Schnittger et al., 2003). *T. separata* is most closely related to *Theileria* sp. (sable) and may be descended from an ancestral *Theileria* infecting wild ruminants (Schnittger et al., 2003).

Sequencing results indicated that the species-specific hybridization signals obtained in the RLB were as a result of cross-reactions of the RLB probes with two novel piroplasm 18S rRNA sequences that are very similar but not identical to those of *Theileria* sp. (sable) and *T. separata*. Similar RLB results were obtained when a novel *Theileria* sp. was found in dogs in South Africa (Matjila et al., 2008). When examining novel piroplasm parasites, therefore, a positive hybridization signal in an RLB should be interpreted with caution, since the probes currently in use may not be truly species-specific.

*Theileria* sp. (sable) has been identified previously using RLB in clinically normal African buffalo (*Syncerus caffer*), various antelope species and cattle (Nijhof et al., 2005; Yusufmia et al., 2010). It may be possible, however, that the positive RLB signals detected in these studies were due to cross-reactions of the *Theileria* sp. (sable) probe with closely related novel piroplasm species and/or genotypes which may not be identical to *Theileria* sp. (sable).

It is uncertain whether the sequences identified in tsessebe represent one or two novel species (or indeed, whether they are distinct from *Theileria* sp. (sable) and *T. separata*), since it is not possible to use 18S rRNA gene sequence variation alone to classify organisms as different species (Chae et al., 1999). There are a similar number of nucleotide differences in the 18S rRNA genes of other distinct and well-characterized piroplasm species, however, e.g. the 18S rRNA genes of *T. parva* and *T. annulata* are 98% identical, with approximately 20 nucleotide differences. Similarly, only nine nucleotide differences distinguish the 18S rRNA gene sequences of *Theileria* sp. (buffalo) and *T. parva* (Sibeko et al., 2008).

All the tsessebe sampled appeared clinically normal and showed no sign of disease post relocation (personal communication, Dr D. Zimmerman). This is typical for many piroplasm infections in wildlife, which do not manifest as clinical disease, unless the host is stressed (Penzhorn, 2006). The *Theileria* species identified in tsessebe may therefore be benign, and infection would thus not result in severe clinical signs or death. The 18S rRNA gene sequences of the new tsessebe parasite/s group closely with *T. separata*, a benign parasite of sheep. A *Theileria* sp., showing a 98.9% similarity to *Theileria* sp. (sable), was regarded as benign in red hartebeest

(*Alcelaphus buseplahus*) (Spitalska et al., 2005), which are closely related to topi (*Damaliscus korrigum*) (Arctander et al., 1999), a congener of the tsessebe. On the other hand, *Theileria* sp. (sable) can cause mortality in roan and sable antelope (Nijhof et al., 2005), which also have a close genetic relation/origin to tsessebe (Matthee and Davis, 2001). The tsessebe parasite could therefore be potentially pathogenic.

The tick species collected, *Rhipicephalus evertsi evertsi*, is widespread and common on livestock throughout much of Africa (Walker et al., 2003) and has been reported from tsessebe in Zimbabwe (Madzingira and Mukaratirwa, 2002). *R. e. evertsi* can transmit *T. separata*, *T. taurotragi*, and *T. equi* (Lawrence et al., 1994; Walker et al., 2003). It is possible, therefore, that this tick could act as a vector of the novel *Theileria* species identified in this study.

Many animals had no or very few ticks on them, although it should be noted that relatively few animals were sampled as many could not be adequately restrained for specimen collection. The low numbers of ticks collected may have been because the capture operation was carried out in winter, although an additional reason may be an innate resistance of tsessebe to tick infestation (Lightfoot and Norval, 1981). Obviously, several other factors such as climatic conditions and host density, will also play a role in determining tick burdens, and hence also the opportunity for infections to be passed on to the host species. The low prevalence of piroplasm infections could be a reflection of the relatively low tick infestations recorded.

Wildlife veterinary practitioners should note the presence of haemoparasites in tsessebe and other wildlife species (Nijhof et al., 2003, 2005, Spitalska et al., 2005, Oosthuizen et al., 2008, 2009) and care should be taken when translocating wild animals, as introduction of the organisms into new areas could cause clinical disease and losses in naïve wildlife and domestic animals, and piroplasm parasite species could become established in areas in which they previously did not occur.

## 5. Acknowledgements

## 6. Conflict of interest statement

None of the authors of this article is aware of any financial or personal relationships with other people or organisations that could inappropriately influence (bias) the work reported here.

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Table 1.  Genus- and species-specific RLB probes used in this study

| Genus / Species | Oligonucleotide probe sequence (5'-3')[a] |
| --- | --- |
| *Ehrlichia/Anaplasma* genus-specific | GGG GGA AAG ATT TAT CGC TA |
| *Anaplasma bovis* | GTA GCT TGC TAT GRG AAC A |
| *Anaplasma centrale* | TCG AAC GGA CCA TAC GC |
| *Anaplasma marginale* | GAC CGT ATA CGC AGC TTG |
| *Anaplasma phagocytophilum* | TTG CTA TAA AGA ATA ATT AGT GG |
| *Ehrlichia canis* | TCT GGC TAT AGG AAA TTG TTA |
| *Ehrlichia chaffeensis* | ACC TTT TGG TTA TAA ATA ATT GTT |
| *Ehrlichia ruminantium* | AGT ATC TGT TAG TGG CAG |
| *Ehrlichia* sp. (Omatjenne) | CGG ATT TTT ATC ATA GCT TGC |
| *Theileria/Babesia* genus-specific | TAA TGG TTA ATA GGA RCR GTT G |
| *Theileria* genus-specific | ATT AGA GTG CTC AAA GCA GGC |
| *Babesia* genus-specific 1 | ATT AGA GTG TTT CAA GCA GAC |
| *Babesia* genus-specific 2 | ACT AGA GTG TTT CAA ACA GGC |
| *Babesia bicornis* | TTG GTA AAT CGC CTT GGT C |
| *Babesia bigemina* | CGT TTT TTC CCT TTT GTT GG |
| *Babesia bovis* | CAG GTT TCG CCT GTA TAA TTG AG |
| *Babesia caballi* | GTG TTT ATC GCA GAC TTT TGT |
| *Babesia canis canis* | TGC GTT GAC GGT TTG AC |
| *Babesia canis rossi* | CGG TTT GTT GCC TTT GTG |
| *Babesia canis vogeli* | AGC GTG TTC GAG TTT GCC |
| *Babesia divergens* | ACT RAT GTC GAG ATT GCA C |
| *Babesia felis* | TTA TGC GTT TTC CGA CTG GC |

| | |
|---|---|
| *Babesia gibsoni* | CAT CCC TCT GGT TAA TTT G |
| *Babesia major* | TCC GAC TTT GGT TGG TGT |
| *Babesia microti* | GRC TTG GCA TCW TCT GGA |
| *Babesia* sp. (sable) | GCG TTG ACT TTG TGT CTT TAG C |
| *Theileria annae* | CCG AAC GTA ATT TTA TTG ATT TG |
| *Theileria annulata* | CCT CTG GGG TCT GTG CA |
| *Theileria bicornis* | GCG TTG TGG CTT TTT TCT G |
| *Theileria buffeli* | GGC TTATTT CGG WTT GAT TTT |
| *Theileria equi* | TTC GTT GAC TGC GYT TGG |
| *Theileria lestoquardi* | CTT GTG TCC CTC CGG G |
| *Theileria mutans* | CTT GCG TCT CCG AAT GTT |
| *Theileria ovis* | TTG CTT TTG CTC CTT TAC GAG |
| *Theileria parva* | GGA CGG AGT TCG CTT TG |
| *Theileria separata* | GGT CGT GGT TTT CCT CGT |
| *Theileria taurotragi* | TCT TGG CAC GTG GCT TTT |
| *Theileria velifera* | CCT ATT CTC CTT TACGAG T |
| *Theileria* sp. (buffalo) | CAG ACG GAG TTT ACT TTG T |
| *Theileria* sp. (kudu) | CTC CAT TGT TTCTTT CCT TTG |
| *Theileria* sp. (sable) | GCT GCA TTG CCT TTT CTC C |
| *Hepatozoon* genus-specific | GCT TTG TAA TTG GAA TGA TAG A |

[a] Symbols used to indicate degenerate positions: R = A/G; W= A/T; Y = C/T.

# Figure Captions

Fig. 1. Neighbor-joining tree showing the phylogenetic relationship of Tsessebe 1 and Tsessebe 2 18S rRNA gene sequences with other *Babesia* and *Theileria* 18S rRNA gene sequences. The tree was rooted using *Prorocentrum micans*, *Sarcocystis muris* and *Toxoplasma gondii* 18S rRNA gene sequences as outgroups. Bootstrap values are shown from 100 replicate trees. The GenBank accession numbers are indicated in parentheses. Branch lengths are proportional to the estimated genetic distance between the strains. The scale bar represents the % nucleotide difference.