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Occurrence of blood-borne tick-transmitted parasites in tsessebe (*Damaliscus lunatus lunatus*) antelope in Vaalbos National Park, Northern Cape Province

by

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Dedication

To Becky and William for the many hours of family time they sacrificed in order to make this possible – thank you. To the rest of my family, in particular my parents for motivating me to become a vet, and to my grandparents, France and Stan, who were an inspiration and naturalists of exception.

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Occurrence of blood-borne tick-transmitted parasites in tsessebe (*Damaliscus lunatus lunatus*) antelope in Vaalbos National Park, Northern Cape Province

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Department: Veterinary Tropical Diseases
Degree: MSc (Veterinary Tropical diseases)

Summary

Blood was collected from 71 tsessebe antelope and ticks from 12 of these animals, in the Vaalbos National Park. The samples were collected when the animals were relocated to a new park as a result of the deproclamation of Vaalbos National Park. DNA was extracted from the blood samples and the V4 hypervariable region of the 18S rRNA gene of any piroplasm parasites present was amplified by PCR. The RLB hybridisation technique was used to identify piroplasm parasites. *Theileria* spp. were identified and full-length 18S rRNA genes were amplified, cloned and sequenced. The results indicated the presence of novel *Theileria* spp. phylogenetically very closely related to both *Theileria* sp. (sable) and *Theileria separata*. The ticks collected were all *Rhipicephalus evertsi evertsi*, which has been shown to be capable of acting as a vector for *Theileria* spp. All animals appeared to be in good health at the time of sampling and after relocation. It is thus possible that, as with several other wildlife species, the *Theileria* spp. identified here do not cause disease under normal circumstances and that a situation of endemic stability exists. Once the host is under any form of stress, however, overt clinical disease may well become evident. The significance of these *Theileria* spp. should not be underestimated,

and care should be taken not to transmit the organisms into new areas. More research will need to be conducted to determine the exact clinical significance of these findings and the role of *Rhipicephalus evertsi evertsi* as a potential vector for these *Theileria* spp.

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Chapter 1. Introduction

Tsessebe antelope (*Damaliscus lunatus*) are listed as endangered in the most recent Red Data Book (CBS Group, EWT & IUCN, 2004 [Electronic resource]). This listing makes the species more threatened than species such as the sable (*Hippotragus niger*) and roan (*Hippotragus equinus*) antelopes, on which much research in this field has already been done.

According to the Red Data book, a taxon is Endangered when the best available evidence indicates that it meets any of several defined criteria, and as a result it is considered to be ***facing a very high risk of extinction in the wild*** *– Red Data Book.*

Decline in tsessebe populations has previously been ascribed mostly to environmental factors – rain and grazing (Dunham, Robertson & Grant, 2004) – as well as competition for grazing with cattle in one case in Zimbabwe (Dunham, Robertson & Swanepoel, 2003). The demise in numbers is cause for concern and reserves are developing strategies in an attempt to explain this phenomenon, and cope with it, notably Kruger National Park (Grant & Van der Walt, 2000).

The purpose of this research project was:

1. To determine whether individuals in a population of tsessebe from Vaalbos National Park are carrying any blood-borne tick-transmitted parasites.
2. If so, to identify these parasites using molecular characterisation.
3. To identify the tick vectors responsible for transmitting these parasites, if possible.

Further studies/research would be required to then establish the effect of such disease on individual animals, and ultimately populations, in terms of morbidity and mortality. These data should then ideally be used to compare various populations (for example Vaalbos National Park, Kruger National Park, and KwaZulu-Natal populations) to see if

the above knowledge can help explain the demise of some populations while others appear to be thriving.

Data from this study can possibly be extrapolated to other species such as roan and sable antelope in the same areas, based on various factors, including their close genetic relation/origin (Matthee & Davis, 2001), and thus their potential similar susceptibility to the same parasites.

This research coincided with the relocation of the majority of the wildlife in the former Vaalbos National Park, to the newly proclaimed Mokala National Park (previously Wintershoek) – a result of a successful land claim and the South African National Parks (SANParks) thus handing Vaalbos National Park over to the claimants and deproclaiming it as a National Park. Vaalbos National Park was based in the Northern Cape Province, south of the Vaal River between Barkly West and Kimberley and was approximately 22 697 ha in size. It was established in 1986 and the habitat comprised a mix of typical Karoo, Kalahari thornveld and grassveld. Endemic small mammals and established populations of springbok and hartebeest flourished in the park, while species such as buffalo and black rhino were reintroduced.

Figure 1.1 – Map indicating the location of Vaalbos National Park

The tsessebe (between 25 and 30 animals) were introduced into Vaalbos National Park and originated from Doorndraai Dam Nature Reserve in the Limpopo Province (at the time of the relocation this province was still named the Transvaal) in the early 1990s – the exact year is not known (Pers. comm. Dr D Zimmerman and Dr M Knight). Doorndraai, which lies just south west of Mokopane (formerly Potgietersrus) and is 7000 ha in size, is also home to other antelope such as sable antelope and mountain reedbuck (*Redunca fulvorufula*) (Renssen, 2006). At the time of the relocation there were no tsessebe in Vaalbos National Park.

Chapter 2. Literature review

It has been known for a long time that tsessebe carry *Theileria* (Carmichael & Hobday, 1975; Stoltsz, 1989), but this *Theileria* sp. has never previously been identified/typed. The role of this *Theileria* species in terms of morbidity and mortality is unknown, although it is reported to have caused death in at least one natural case in a young tsessebe (Jardine, 1992).

Piroplasms indistinguishable from those of *Theileria* spp. have been noted in several wild antelope in South Africa, including greater kudu (*Strepsiceros strepsiceros*), steenbok (*Raphicerus campestris*), waterbuck (*Kobus ellipsiprymnus*), reedbuck (*Redunca arundinum*), mountain reedbuck, bushbuck (*Tragelaphus scriptus*), sable antelope, blue wildebeest (*Connochaetes taurinus*) and grey duiker (*Sylvicapra grimmia*) (Neitz, 1957). Brocklesby and Vidler (1965) stated that *Theileria* spp. were also found in East Africa in a variety of antelope (including gazelle, hartebeest, wildebeest and eland) and other species. Reporting on endoparasites of various East African vertebrates, Peirce (1972) stated that because of the difficulty in distinguishing between *Babesia*, *Theileria* and *Nuttalia* spp. in mild infections, all such organisms were simply listed as piroplasm infections in his article. He confirmed finding piroplasms in the eland (*Taurotragus oryx*), Grant's gazelle (*Gazella granti*), Thomson's gazelle (*Gazella thomsoni*), hartebeest (*Alcephalus buselaphus cokii*) and the blue wildebeest in East Africa. In a study carried out in Botswana in which blood smears were made from a total of 282 wild Bovidae, Carmichael and Hobday (1975) reported theilerial piroplasms in tsessebe (18 animals sampled) and seven other species: African buffalo (*Syncerus caffer*), impala (*Aepyceros melampus*), blue wildebeest, eland, lechwe (*Kobus leche*), greater kudu and sable. Piroplasms indistinguishable from those of *Theileria* spp. were also noted in gemsbok (*Oryx gazella*) and tsessebe by Stoltsz (1989). Eland are also known to be susceptible to *Theileria* in the form of *T. taurotragi* (Lawrence, De Vos & Irvin, 1994; Du Toit, 2005).

The development and use of species-specific probes in combination with reverse line blot hybridization has taken these observations a step further and proved beyond doubt that

pathogen DNA isolated from roan antelope, sable antelope, greater kudu, and grey duiker in South Africa, were all in fact *Theileria* spp. (Nijhof, Pillay, Steyl, Prozesky, Stoltsz, Lawrence, Penzhorn & Jongejan, 2005). Spitalska, Riddell, Heyne and Sparagano (2005) also used polymerase chain reaction (PCR) and reverse line blot (RLB) to isolate *Theileria* spp. from red hartebeest (*Alcelaphus buselaphus caama*) and *Rhipicephalus evertsi mimeticus* ticks retrieved from the red hartebeest, in Namibia. The *Theileria* sp. from red hartebeest was characterized by sequencing and did not match any of the usual African *Theileria* species (*T. parva, T. mutans, T. taurotragi, T. annulata* and *T. velifera*) but showed a 98.9% similarity to Genbank accession number L19081 (sequence published in 1994 by Allsopp, Cavalier-Smith, De Waal and Allsopp, and described initially as a *Cytauxzoon*) and the srRNA gene sequence alignment indicated that it is most closely related to *T. buffeli* (GenBank accession number Z15106; similarity 96%). Phylogenetic studies have shown the red hartebeest to be similar to the topi (*Damaliscus lunatus*), of which the tsessebe (*Damaliscus lunatus lunatus*) is considered a subspecies (Arctander, Johansen & Coutellec-Vreto, 1999). These findings are thus the closest anyone has come to doing molecular characterisation of blood-borne parasites on tsessebe to date.

There has been no previous detailed research of this nature (using molecular characterisation to type *Theileria*, or other blood-borne parasites) on the tsessebe. Thus it is possible that earlier observations and records may in fact not have been true *Theileria* infections, but piroplasms of another organism.

What is also previously unknown is whether there are other blood-borne parasites carried by tsessebe, and what role such parasites might play in terms of disease, and thus ultimately on individual animals and the greater population in terms of morbidity and mortality. The role of the tick vector has also not been established in this regard.

Penzhorn (2006) summarises *Babesia* infections in wild animals very well and highlights several species that have been shown to be susceptible under various conditions, including several wild carnivore species, elephant (*Loxodonta africana*), black rhino

(*Diceros bicornis*), sable antelope, bison (*Bison bison*), hyraxes (Procaviidae family) and several deer species (Cervidae family). There is no mention of *Babesia* in tsessebe, however.

The susceptibility of tsessebe to *Ehrlichia ruminantium* is also still unknown, despite results from a clinical trial in which tsessebe were experimentally inoculated with in vitro culture-derived *E. ruminantium* organisms. The tsessebe seroconverted, but disease could not be spread to susceptible sheep by the tick vector *Amblyomma hebraeum*, after two attempts, thus leaving the susceptibility of tsessebe to this disease undetermined (Peter, Anderson, Burridge, Perry & Mahan, 1999). In another study in Zimbabwe, however, blood and bone marrow specimens from tsessebe were shown to be positive for *E. ruminantium* using a PCR assay based on the *E. ruminatium* map1 gene (Kock, Vanvliet, Charlton & Jongejan, 1995). This may have had to do with seroconversion, however, and does not prove that transmission to other susceptible hosts could take place from these infected animals.

Chapter 3. Materials and methods

As part of the above-mentioned relocation operation, all the tsessebe (n = 215) in Vaalbos National Park were caught by the SANParks Southern Veterinary Services Team using mass-capture techniques, and were handled for the purposes of tranquilisation. Sampling was focused on two days of capture. Blood was collected from 71 tsessebe (27 collected on 10/07/2006 and 44 collected on 17/09/2006) by bleeding the jugular vein using the "Vacutainer" system (EDTA and serum samples). This represents 33.0% of the total relocated population. Animals sampled included a mix of ages and sex – unfortunately these data were not recorded at the time of sample collection. The blood samples were stored on ice in the field, and then in a conventional fridge until being transported to Pretoria on ice.

Ticks were collected from twelve of these animals, thus making the sample totally random. All larvae and nymphs can be assumed to be collected from the ears and adults from under the tail. The ticks were stored in alcohol-filled tubes and were identified using a dissection microscope.

Blood samples were analysed in the laboratory of the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, Onderstepoort, by making use of the RLB hybridization technique, as described by Nijhof *et al*. (2005).

DNA extraction and Polymerase Chain Reaction (PCR) amplification

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: 20 µl proteinase K (20 mg/ml) was pipetted into a 1.5 ml tube and 200 µl of blood and 180 µl of buffer AL were added and the sample was then subjected to vortex. Thereafter the sample was incubated at 56 ºC for 10 minutes and then centrifuged. 200 µl 100% ethanol was added and the sample was again subjected to vortex before transferring the sample into the QIAamp mini spin column. The sample was then centrifuged at full speed for 1 minute and the flow through discarded. The

column was placed back in the 2 ml collection tube and 500 µl buffer AW1 added, before centrifuging for 1 minute at 8000 rpm, and thereafter discarding the flow through. The washing step was repeated, this time adding 500 µl buffer AW2. Then the column was placed in a new 2 ml collection tube and centrifuged for 1 minute at full speed to remove residual AW buffer. The column was then placed in a 1.5 ml tube and 100 µl elution buffer AE added directly onto the DNeasy membrane. The column was incubated at room temperature for 1 minute and centrifuged for 1 minute at 8000 rpm to elute the DNA. Additional centrifugation was then carried out at full speed for 1 minute to optimize DNA recovery. DNA samples were then aliquoted and 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, 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/Ehrlichia* species, were used to amplify the V4 variable region of the parasite 18S rRNA gene as previously described (Gubbels, De Vos, Van Der Weide, Viseras, Schouls, De Vries & Jongejan, 1999). The primers (20 pmol/µl) at 0.25 µl per reaction, UDG master mixture at 12.5 µl per reaction, and molecular grade water at 9.5 µl per reaction, were added together to make the PCR reaction master mix. 22.5 µl of the master mix was aliquoted into each PCR tube. 2.5 µl of extracted template DNA was added into each PCR tube and mixed by pippeting. The tubes were then placed in the preheated programmed thermocycler and subjected to the thermal cycling conditions shown in Table 3.1.

Table 3.1 – Thermocycler programme

Number of cycles	**Time**	**Temperature**	**Purpose**
1 cycle	3 min	37 °C	Activate UDG
1 cycle	10 min	94 °C	Inactivate UDG & activate *Taq* polymerase
2 cycles	20 sec	94 °C	Denaturing of template DNA
2 cycles	30 sec	67 °C	Annealing of primers
	30 sec	72 °C	Extending of PCR products by *Taq*

			polymerase
2 cycles	20 sec	94 °C	
	30 sec	65 °C	
	30 sec	72 °C	
2 cycles	20 sec	94 °C	
	30 sec	63 °C	
	30 sec	72 °C	
2 cycles	20 sec	94 °C	
	30 sec	61 °C	
	30 sec	72 °C	
2 cycles	20 sec	94 °C	
	30 sec	59 °C	
	30 sec	72 °C	
40 cycles	20 sec	94 °C	
	30 sec	57 °C	
	30 sec	72 °C	
1 cycle	7 min	72 °C	Final extension
		4 °C	Hold

Reverse Line Blot (RLB) hybridization

RLB hybridization was performed as described by Nijhof *et al.* (2005). The membrane was incubated for 5 minutes in 10 ml 2 X SSPE/0.1% SDS at room temperature. PCR products were diluted by mixing 10 µl PCR product in 140 µl 2 X SSPE/0.1% SDS. Diluted PCR products were then denatured for 10 minutes at 96 °C in a heating block and cooled on ice immediately afterwards. Once cool, samples were briefly centrifuged. The membrane was placed in an MN45 miniblotter apparatus (Isogen Life Science, Maarssen, The Netherlands) and residual fluid removed by aspiration. The slots were then filled with diluted PCR product, being careful to avoid air bubbles. Empty lanes were filled with 2 X SSPE/0.1% SDS to avoid cross flow. The membrane was then hybridized at 42 °C for 60 minutes on a horizontal surface. Afterwards the samples were removed by aspiration and the membrane removed from the blotter.

The membrane was washed twice in preheated 2 X SSPE/0.5% SDS for 10 minutes, at 50 °C, in a water bath, while shaking gently. The membrane was then incubated with 100 ml 2 X SSPE/0.5% SDS and 25 µl peroxidase-labeled streptavidin conjugate (1.25 U) for 30 minutes at 42 °C. After this, the membrane was washed twice in preheated 2 X SSPE/0.5% SDS for 10 minutes, at 42 °C, in a water bath, while shaking gently. The

membrane was then washed twice with 2 X SSPE for 5 minutes at room temperature, while shaking gently, and then transferred onto an overhead sheet. 10 ml premixed ECL (enhanced chemiluminescence detection fluid) solution (5 ml ECL 1 plus 5 ml ECL 2) was then spread over the membrane and the membrane covered with an overhead sheet. Air bubbles were removed by gently rolling a tube over the sheet and then the membrane was incubated for 1 minute at room temperature. The membrane was then placed between two clean overhead sheets and placed in a radiography exposure cassette. The X-ray film was exposed for 10 minutes and then developed.

The original samples were subjected to this process in two batches, unfortunately on different blots due to a laboratory oversight. A key probe, *Babesia* sp. (sable), was missing from the second blot and thus it was decided to submit all positive samples from the first two blots, to a third RLB, with all the necessary probes. The probes used for the three blots are listed in Table 3.2.

Table 3.2 – Probes used on the three respective blots during the RLB process

Blot 1 Probes	**Blot 2 Probes**	**Blot 3 Probes**
Ehrlichia/Anaplasma catch-all	*Ehrlichia/Anaplasma* catch-all	*Ehrlichia/Anaplasma* catch-all
A. centrale	*A. centrale*	*A. centrale*
A. marginale	*A. marginale*	*A. marginale*
A. phagocytophilum	*A. phagocytophilum*	*A. phagocytophilum*
	A. phagocytophilum	
	A. phagocytophilum	
	A. phagocytophilum	
E. ruminantium	*E. ruminantium*	*E. ruminantium*
A. bovis	*A. bovis*	*A. bovis*
E. chaffeensis	*E. chaffeensis*	*E. chaffeensis*
Ehrlichia sp. (Omatjenne)	*Ehrlichia* sp. (Omatjenne)	*Ehrlichia* sp. (Omatjenne)
E. canis	*E. canis*	*E. canis*

Theileria/Babesia catch all	*Theileria/Babesia* catch all	*Theileria/Babesia* catch all
Theileria catch-all		*Theileria* catch-all
Babesia catch-all 1		*Babesia* catch-all 1
Babesia catch-all 2		*Babesia* catch-all 2
B. felis	*B. felis*	*B. felis*
B. divergens	*B. divergens*	*B. divergens*
B. microti	*B. microti*	*B. microti*
B. bigemina	*B. bigemina*	*B. bigemina*
B. bovis	*B. bovis*	*B. bovis*
B. rossi	*B. rossi*	*B. rossi*
B. canis canis	*B. canis canis*	*B. canis canis*
B. canis vogeli	*B. canis vogeli*	*B. canis vogeli*
B. major	*B. major*	*B. major*
B. bicornis	*B. bicornis*	*B. bicornis*
B. caballi	*B. caballi*	*B. caballi*
B. gibsoni		*B. gibsoni*
Babesia sp. (sable)		*Babesia* sp. (sable)
Babesia sp. (cheetah)		
B. leo		
Theileria sp. (kudu)	*Theileria* sp. (kudu)	*Theileria* sp. (kudu)
Theileria sp. (sable)	*Theileria* sp. (sable)	*Theileria* sp. (sable)
T. bicornis	*T. bicornis*	*T. bicornis*
T. annulata	*T. annulata*	*T. annulata*
T. buffeli	*T. buffeli*	*T. buffeli*
Theileria sp. (buffalo)	*Theileria* sp. (buffalo)	*Theileria* sp. (buffalo)
T. mutans	*T. mutans*	*T. mutans*
T. parva	*T. parva*	*T. parva*
T. taurotragi	*T. taurotragi*	*T. taurotragi*
T. velifera	*T. velifera*	*T. velifera*
T. equi	*T. equi*	*T. equi*

T. lestoquardi	*T. lestoquardi*	*T. lestoquardi*
T. separata		*T. separata*
T. ovis		*T. ovis*
T. annae		*T. annae*
		Hepatozoon

Cloning and sequencing

Two samples positive for *Theileria* sp. (sable) and *Theileria separata* (samples 22 and 23 collected 2006/09/17; referred to as B22 and B23 in lab) were selected for further characterisation to verify results. The other four positive samples did not contain adequate DNA for this process. The same process as described by Oosthuizen, Zweygarth, Collins, Troskie & Penzhorn (2008) was followed.

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'). The amplification mixture contained 2.5 µl DNA (~75 ng), 12.5 µl Expand high-fidelity PCR master mix (Roche Diagnostics, Mannheim, Germany), 0.1 µM of each primer, and nuclease-free water to a total volume of 25 µl. For each of the samples, the reaction was duplicated four times to obtain a total volume of 100 µl. Amplification was performed in an automated thermocycler (Perkin-Elmer, Foster City, CA). The PCR cycling conditions are shown in Table 3.3. The amplification products were stored at 4°C.

Table 3.3 – PCR cycling conditions

Number of cycles	**Time**	**Temperature**	**Purpose**
1 cycle	2 min	94 °C	Initial denaturation of template DNA
40 cycles	30 sec 1 min 1 min	94 °C 55 °C 72 °C	Denaturing of template DNA Annealing of primers Extending of PCR products by *Taq* polymerase
1 cycle	7 min	72 °C	Final extension
		4 °C	Hold

The PCR amplicons obtained from the four duplicate reactions were pooled in order to minimize the possibility of obtaining sequence errors originating early in any one of the reactions, and purified using the QIAquick PCR purification kit (Blood and Body Fluid Spin Protocol) (QIAGEN and Southern Cross Biotechnologies, SA). This was done by adding 500 µl (5 volumes to 1 volume of the PCR sample) of Buffer PB to the PCR sample and then mixing. A QIAquick spin column was then placed in a 2 ml collection tube and the sample added to the column and centrifuged at 13 000 rpm for 30-60 seconds to bind the DNA. The flow through was discarded and the sample returned to the collection tube for washing by adding 750 µl Buffer PE to the column and again centrifuging at 13 000 rpm for 30-60 seconds. Again the flow through was discarded and the column placed back in the collection tube and centrifuged at 13 000 rpm for 60 seconds. The column was then placed in a clean 1.5 ml microcentrifuge tube and the DNA eluted by adding 20 µl Buffer EB to the centre of the QIAquick membrane and letting the column stand for 1 minute. Then the column was centrifuged for 1 minute.

Purified PCR products of samples B22 and B23 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). For each sample, three 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') as well as the RLB-F2 and RLB-R2 primers described before. Sequencing was performed at Inqaba Biotechnical Industries [Pty] Ltd using a SpectruMedix model SCE 2410 automated sequencer (SpectruMedix, State College, PA).

Sequences were assembled and edited using GAP4 of the Staden package (version 1.6.0 for Windows) (Staden, Beal & Bonfield, 2000). Blastn (Altschul, Gish, Miller, Myers & Lipman, 1990) was used to search the public sequence databases for homologous sequences. The assembled sequences were aligned with published sequences of related genera by using CLUSTAL W (Thompson, Higgins & Gibson, 1994). 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). This model takes transitional and transversional substitution rates into account. The Jukes and Cantor (1969) correction model was also applied for multiple base changes. Phylogenetic trees were constructed with the MEGA 3.0 software package (Kumar, Tamura & Nei, 2004) using both the neighbor-joining (Saitou & Nei, 1987) and maximum-parsimony methods. Bootstrapping was applied using 1,000 replicates/tree for the distance method and 100 replicates/tree for the parsimony method (Felsenstein, 1985). The two different substitution models were used in order to identify clades supported by the highest possible bootstrap values.

Chapter 4. Results

RLB hybridization revealed that ten of the 71 samples collected were positive for a *Theileria* spp., a 14% incidence in the sample group (Table 4.1). Although some positive signals were faint, they were definite positives.

Looking at the first two blots (i.e. the first test for each sample), all of the ten positive samples were positive on the *Theileria/Babesia* catch-all probe, with eight of the ten positives also positive for *Theileria* sp. (sable). Of these eight samples, five were also positive on the *Babesia* A1 catch-all probe, and two of these five were also positive on the *Theileria separata* probe (Table 4.1).

Table 4.1 - Results of blot 1 and blot 2

Sample ID	**Blot**	**Collection date**	***Theileria/ Babesia* catch-all**	***Babesia* catch-all – A1 (not on blot 2)**	***Theileria* sp. (sable)**	***T. separata***
11	2	2006/07/10	Y		Y	
12	2	2006/07/10	Y			
13	2	2006/07/10	Y		Y	
22	1	2006/09/17	Y	Y *	Y	
23	1	2006/09/17	Y	Y	Y	Y *
25	1	2006/09/17	Y	Y	Y	Y
28	1	2006/09/17	Y	Y *	Y	
33	1	2006/09/17	Y	Y	Y	
38	1	2006/09/17	Y *			
44	2	2006/09/17	Y		Y	
All other samples were negative.						

Y: positive for this probe

Y*: very faint signal

After some inconsistent results in the laboratory and since the *Babesia* sp. (sable) probe was missing from blot number 2, it was decided to repeat the RLB for all of the positive

samples on blot number 3 with all the necessary probes on it. The results changed somewhat (Table 4.2). There were no *Babesia* positive signals for any samples, and the first three samples did not test positive on any *Theileria*, or other, probes either. However, six of the ten samples were still positive on the *Theileria/Babesia* catch-all probe, as well as on the *Theileria* catch-all probe and the *Theileria* sp. (sable) probe. Three of these latter samples were also positive on the *Theileria separata* probe. This reduced the percentage of positive samples in the sample group to an 8.5 % incidence. It is important to note that neither blot 1 nor blot 2 had a *Theileria* catch-all probe present, thus comparisons in this regard are not possible.

Table 4.2 – Results of blot 3 (repeat of the RLB for all previously positive samples)

Sample ID	**Collection date**	***Theileria/ Babesia* catch-all**	***Babesia* catch-all – A1**	***Theileria* catch-all**	***Theileria* sp. (sable)**	***T. separata***
11	2006/07/10	-			-	
12	2006/07/10	-				
13	2006/07/10	-			-	
22	2006/09/17	Y	-	Y	Y	**+**
23	2006/09/17	Y	-	Y	Y	Y
25	2006/09/17	Y	-	Y	Y	Y
28	2006/09/17	Y	-	Y	Y	
33	2006/09/17	Y	-	Y	Y	
38	2006/09/17	Y		**+**	**+**	
44	2006/09/17	-		-	-	

-: represents where a positive result was previously obtained, now negative

+: positive for this probe where a negative result was previously obtained

Y: positive for this probe on both occasions

Purified PCR products of samples B22 and B23 (samples 22 & 23 collected on 2006/09/17) were cloned and sequenced, and yielded the following results:

1) Sequences were obtained from 3 clones of sample B22 and the final edited length of these samples was B22-11 – 1662bp, B22-26 -1662 bp, and B22-27 – 1575bp.
2) Sequences were obtained from 2 clones of sample B23 and the final edited length of these samples was B23-6 – 1661 bp and B23-5 – 1662bp.

Blast results of these samples produced the following:

Table 4.3 – Blast result (same length as in tree) of sample B23-6: 1543 bp

Accession	Description	Max score	Total score	Query coverage	E value	Max ident	Links
EU053201.1	*Theileria* sp. VT12 18S ribosomal RNA gene, partial sequence	2828	2828	100%	0.0	99%	
EU053200.1	*Theileria* sp. BC295 18S ribosomal RNA gene, partial sequence	2828	2828	100%	0.0	99%	
EU053199.1	*Theileria* sp. BC281 18S ribosomal RNA gene, partial sequence	2828	2828	100%	0.0	99%	
L19081.1	*Theileria* sp. 18S ribosomal RNA	2800	2800	100%	0.0	99%	
AY735116.1	*Theileria* sp. dama gazelle clone 55 small subunit ribosomal RNA gene, complete sequence	2726	2726	100%	0.0	98%	
AY735115.1	*Theileria* sp. dama gazelle clone 11 small subunit ribosomal RNA gene, complete sequence	2726	2726	100%	0.0	98%	
AY260175.1	*Theileria separata* 18S ribosomal RNA gene, complete sequence	2723	2723	100%	0.0	98%	
AF529272.1	*Theileria* sp. ex Yamaguchi Sika Deer isolate 52 18S ribosomal RNA gene, partial sequence	2651	2651	100%	0.0	97%	
AF529273.1	*Theileria* sp. ex Yamaguchi Sika Deer isolate 47 18S ribosomal RNA gene, partial sequence	2651	2651	100%	0.0	97%	
AF529271.1	*Theileria* sp. ex Yamaguchi Sika Deer isolate 22 18S ribosomal RNA gene, partial sequence	2634	2634	100%	0.0	97%	
AY748466.1	*Theileria* sp. grey duiker 5 18S ribosomal RNA gene, partial sequence	2634	2634	98%	0.0	97%	

Table 4.4 – Blast result (same length as in tree) of sample B22-11: 1544 bp

Accession	Description	Max score	Total score	Query coverage	E value	Max ident	Links
EU053201.1	*Theileria* sp. VT12 18S ribosomal RNA gene, partial sequence	2795	2795	100%	0.0	99%	
EU053200.1	*Theileria* sp. BC295 18S ribosomal RNA gene, partial sequence	2795	2795	100%	0.0	99%	
EU053199.1	*Theileria* sp. BC281 18S ribosomal RNA gene, partial sequence	2795	2795	100%	0.0	99%	
L19081.1	*Theileria* sp. 18S ribosomal RNA	2769	2769	100%	0.0	99%	
AY260175.1	*Theileria separata* 18S ribosomal RNA gene, complete sequence	2750	2750	100%	0.0	98%	
AY735116.1	*Theileria* sp. dama gazelle clone 55 small subunit ribosomal RNA gene, complete sequence	2743	2743	100%	0.0	98%	
AY735115.1	*Theileria* sp. dama gazelle clone 11 small subunit ribosomal RNA gene, complete sequence	2743	2743	100%	0.0	98%	
AY748466.1	*Theileria* sp. grey duiker 5 18S ribosomal RNA gene, partial sequence	2630	2630	98%	0.0	97%	

Table 4.5 – Blast result of sample B23-6: 1661 bp (full edited sequence)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident	Links
L19081.1	*Theileria* sp. 18S ribosomal RNA	3018	3018	100%	0.0	99%	
EU053201.1	*Theileria* sp. VT12 18S ribosomal RNA gene, partial sequence	2983	2983	97%	0.0	99%	
EU053200.1	*Theileria* sp. BC295 18S ribosomal RNA gene, partial sequence	2983	2983	97%	0.0	99%	
EU053199.1	*Theileria* sp. BC281 18S ribosomal RNA gene, partial sequence	2983	2983	97%	0.0	99%	
AY735116.1	*Theileria* sp. dama gazelle clone 55 small subunit ribosomal RNA gene, complete sequence	2939	2939	100%	0.0	98%	
AY260175.1	*Theileria separata* 18S ribosomal RNA gene, complete sequence	2935	2935	100%	0.0	98%	
AY735115.1	*Theileria* sp. dama gazelle clone 11 small subunit ribosomal RNA gene, complete sequence	2933	2933	100%	0.0	98%	

Table 4.6 – Blast result of sample B22-11: 1661 bp (full edited sequence)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident	Links
L19081.1	*Theileria* sp. 18S ribosomal RNA	2987	2987	100%	0.0	99%	
AY260175.1	*Theileria separata* 18S ribosomal RNA gene, complete sequence	2963	2963	100%	0.0	98%	
AY735116.1	*Theileria* sp. dama gazelle clone 55 small subunit ribosomal RNA gene, complete sequence	2955	2955	100%	0.0	98%	
EU053201.1	*Theileria* sp. VT12 18S ribosomal RNA gene, partial sequence	2950	2950	97%	0.0	99%	
EU053200.1	*Theileria* sp. BC295 18S ribosomal RNA gene, partial sequence	2950	2950	97%	0.0	99%	
EU053199.1	*Theileria* sp. BC281 18S ribosomal RNA gene, partial sequence	2950	2950	97%	0.0	99%	
AY735115.1	*Theileria* sp. dama gazelle clone 11 small subunit ribosomal RNA gene, complete sequence	2950	2950	100%	0.0	98%	

Phylogenetic trees were constructed and the results are shown in Figs 4.1 and 4.2.

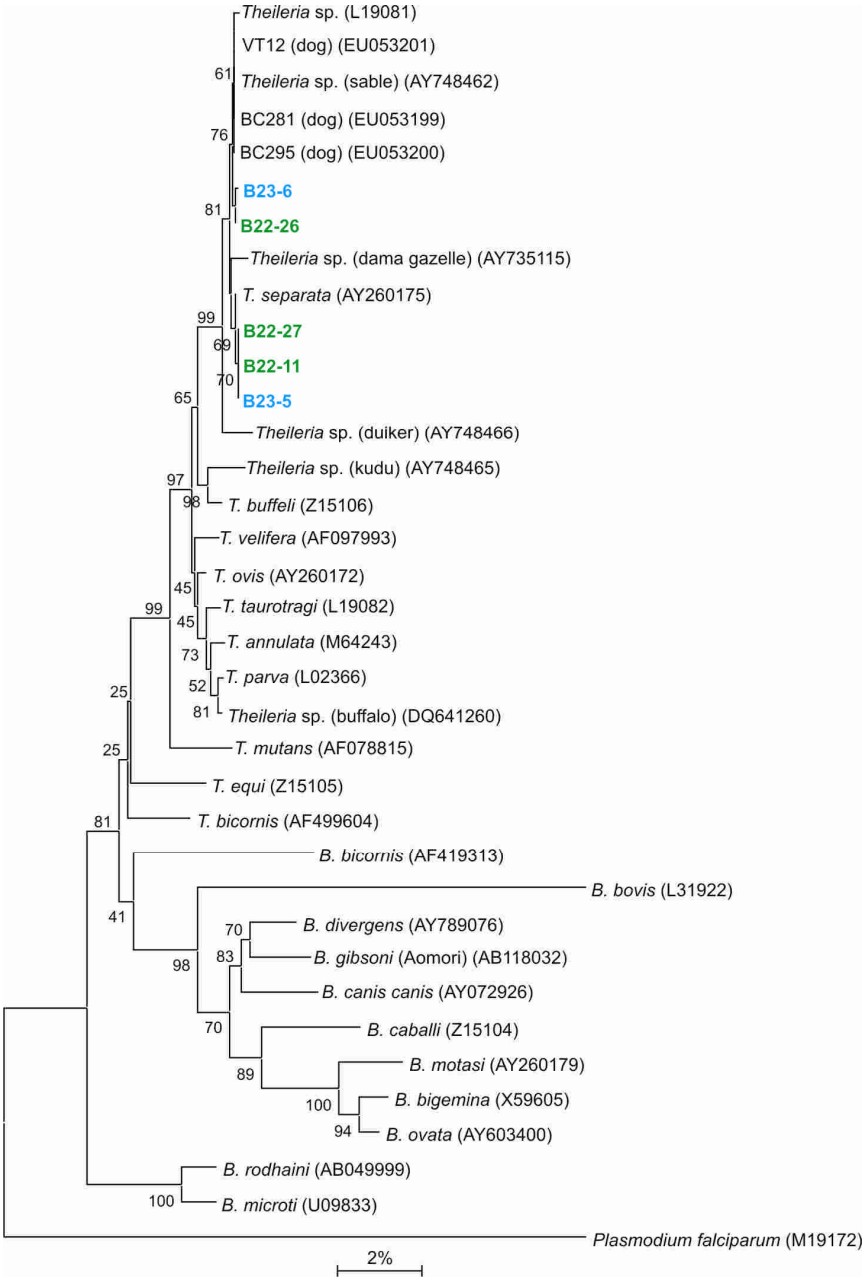

Figure 4.1 – Neighbour-joining tree showing the phylogenetic relationship of the *Theileria* spp. identified in tsessebe with other *Theileria* and *Babesia* species, based on the 18S rRNA gene sequences. Branch lengths are proportional to the estimated genetic distance between the strains and indicated by the scale below the tree. Vertical lengths are not significant.

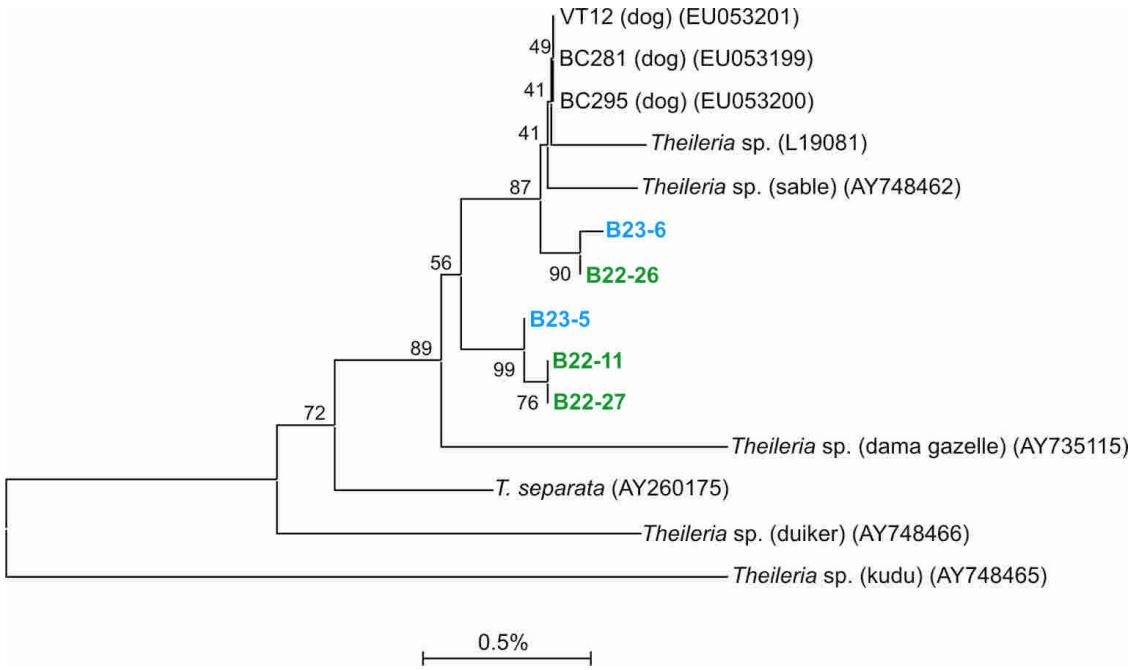

Figure 4.2 – Neighbour-joining tree showing the phylogenetic relationship of the *Theileria* spp. identified in tsessebe with closely related *Theileria* species, based on the 18S rRNA gene sequences. Branch lengths are proportional to the estimated genetic distance between the strains and indicated by the scale below the tree. Vertical lengths are not significant.

Tick samples were collected from 12 of the 71 animals. All ticks collected were identified as *Rhipicephalus evertsi evertsi* using a dissection microscope, and a break down of numbers and adults, larvae and nymphs can be seen in Table 4.7.

Table 4.7 – Summary of ticks collected

Sample ID	**Collection date**	**Tick species - *Rhipicephalus evertsi evertsi***	**Larvae**	**Nymphs**	**Adults**
1	2006/07/10	Y	12	19	0
2	2006/07/10	Y	11	4	0
3	2006/07/10	Y	51	11	0
4	2006/07/10	Y	19	9	0

5	2006/07/10	Y	59	17	0
6	2006/07/10	Y	14	19	0
11	2006/07/10	Y	16	7	0
12	2006/07/10	Y	18	2	0
13	2006/07/10	Y	1	2	0
22	2006/07/10	Y	0	0	1 F
23	2006/07/10	Y	0	0	3 M
25	2006/07/10	Y	0	0	2 (1F, 1M)

F: Female

M: male

Chapter 5. Discussion

The discovery of *Theileria* spp. in tsessebe is no surprise, but the findings of this research prove beyond doubt that these organisms do infect tsessebe. More specifically, this research proves that a *Theileria* sp., or *Theileria* spp., closely related to *Theileria* sp. (sable) and *Theileria separata* can infect tsessebe, and occur in this geographical area.

It is generally accepted that PCR, RLB and DNA sequencing are very accurate methods that provide us with reliable results, provided laboratory technique has been accurate. These techniques have highlighted gross inconsistencies in the original taxonomic characterisation methods, which were based on observations of morphology and certain general phenotypic characteristics (Allsopp & Allsopp, 2006).

There are several discrepancies between the first set of results and the final set, but these can probably be best explained by laboratory inconsistencies at the time, leading to contamination/false positives. This statement is primarily based on results of other work done in the laboratory at the same time which were also inconsistent. The final RLB on blot 3 tested all samples that were positive for any probe in the initial testing, and left us with only six positive samples, all from the original blot 1 (probably co-incidental). When this final RLB was run, laboratory results were back to normal, and it is thus believed there were no inconsistent results, making these results the definitive ones for discussion and conclusion purposes.

While laboratory inconsistencies are ascribed as the most likely cause of the changed results, it is possible that the original positive results were in fact correct, and that on the second test no parasitic DNA was extracted in the sample tested, thus giving a negative result. This is an ongoing debate when inconsistent results are obtained using PCR and RLB, but as long as the lab technique is judged to have been correct, and there is no known cross reaction possible, then it is generally accepted that any positive results were in fact positive. In this case there were other inconsistent laboratory results, which lead to

the conclusion that there was a problem with the laboratory technique, and hence why I would not consider the original results as true positives.

It might also be possible that there are unknown factors that affected the samples and thus resulted in negative testing, in particular for *Babesia*, in the second round of tests. The latter is unlikely, however, since the samples were stored frozen, in the standard manner.

Several of the reports found in the literature refer to piroplasms that are suspected to be theilerial organisms, or that cannot be distinguished from *Babesia* and/or *Nuttalia* spp. Thus these may never have been true *Theileria*, or conversely they may have in fact been *Theileria* sp. (sable) or *T. separata*, or some other *Theileria* sp. for that matter.

There is thus no previous record of *T. separata* infecting any other antelope species, let alone tsessebe. *Theileria separata* is known to normally be a benign infection of sheep, transmitted by *R. evertsi evertsi*, and is considered endemic in Southern Africa (Lawrence *et al.* 1994). Based on their research, Schnittger, Yin, Gubbels, Beyer, Niemann, Jongejan and Ahmed (2003) stated that the independent evolution of almost all sheep/goat piroplasms suggests that speciation may have occurred after transfer of the piroplasm-transmitting tick from a primal wild ruminant host to domestic sheep and goats. This suggests that *T. separata* may in fact have been a *Theileria* infecting wild ruminants originally. Based on this, it should be no surprise we found very similar parasites in the tsessebe. *Theileria separata* was not sequenced in this study, but perhaps this should be done in future to determine if this *T. separata* is in fact 100% identical to *T. separata* infecting sheep in South Africa. Only then can further comment be made, or more specific conclusions drawn.

With the advent of RLB, Nijhof *et al.* (2005) not only typed several new *Theileria* spp. from wildlife in fatal cases, but also found clinically healthy animals of several different ruminant species to be positive for *Theileria* sp. (sable). These species include the African buffalo, African short-horn cattle (*Bos indicus*), blesbok (*Damaliscus pygargus phillipsi*), blue wildebeest, klipspringer (*Oreotragus oreotragus*), and reedbuck. All

samples originated in South Africa (except for the blesbok which originated in Swaziland, and the African short-horn cattle which originated in Tanzania), but none of their samples came from the same region as the tsessebe tested in this study. Thus finding *Theileria* sp. (sable) in apparently healthy tsessebe is no great surprise. Both these studies indicate a wide distribution of *Theileria* sp. (sable).

Even though six samples tested positive for *Theileria* sp. (sable) in the final test, it was decided to clone and sequence some of the positive samples to determine if this *Theileria* parasite is in fact 100% identical to *Theileria* sp. (sable), previously isolated from other species of antelope, or if there are any differences. This is particularly significant in view of the fact that this parasite has been shown to be pathogenic in other antelope species (Nijhof *et al*. 2005). The results showed that there is a difference of only one base pair between the *Theileria* spp. isolated here, and the *Theileria* sp. (sable) previously on record with GenBank (accession number: AY748462). However, both samples (B22 & B23) sequenced showed between 97% and 100% match with seven other *Theileria* spp. already identified and on record, using the Blastn package (Altschul *et al.* 1990). This is a higher match than compared to the match with *Theileria* sp. (sable). More specifically one of the *Theileria* spp. showing a 100% match to both sequenced samples is *T. separata* (accession number: AY260175). Are we thus not dealing with essentially the same organism in all the positive samples, and because of its similarity to both *Theileria* sp. (sable) and *T. separata*, it has bound with both probes? A similar situation occurs with *Theileria* sp. (buffalo) and *T. parva* and a melting curve had to be used to differentiate between the two species on real-time PCR assay. This is because both were competing for the amplification primer because of their similar 18S rRNA gene sequence (Sibeko, Oosthuizen, Collins, Geysen, Rambritch, Latif, Groeneveld, Potgieter & Coetzer, 2008). Does this suggest a true new species, or simply indicate a process of dynamic evolutionary diversification in which clear species separation has not yet been completed? The latter would explain why some *Theileria* parasites, for example those belonging to the *T. buffeli* group, cannot be clearly separated into different species (Chae, Allsopp, Waghela, Park, Kakuda, Sugimoto, Allsopp, Wagner & Holman, 1999).

Unfortunately, there is no general consensus on the extent of sequence variation which must be present in the 18S rNA genes in order to consider organisms as a different species. Schnittger *et al.* (2003) proposed a method of using minimum and maximum threshold values to compare different species, and in this way consider species similar or separate species. This method was revised using the parasites' biological characteristics and compared with known classic taxonomy, and could be reconciled, thus verifying the validity of their applied decision-making procedure. A similar procedure may be useful in this instance.

No matter how you classify these *Theileria*, the fact is that they are definitely *Theileria* parasites, and in my opinion, the effect they have on the host species is a more important consideration at this stage. All the animals sampled appeared clinically healthy at the time of collection, and showed no sign of disease post relocation (personal communication Dr David Zimmerman, SANParks veterinarian in charge of the operation, 2006). This is typical for many protozoan infections in wildlife which do not manifest as clinical disease, unless conditions of stress exist (such as nutritional stress, relocation stress, confinement, competition, etc.). In this particular case, even with relocation stress, no disease was evident. This could simply be because of a good resistance/immune system in the infected individuals, or the fact that the stress suffered was not adequate to suppress the immune system to a level where disease would result. The tsessebe were captured in a mass-capture boma, loaded onto trucks and briefly handled for sampling and sedation, and then transported a few hours before being released directly onto the new reserve. This method of relocation ensures minimal stress and may be the main reason why no disease was noted subsequent to relocation. It should be emphasized at this point that no fatalities were noted and mild clinical disease may thus have been present, but typically *Theileria* clinical infections in wildlife result in death, and thus it is assumed that no disease developed (Nijhof *et al*. 2005). The new reserve is also large and the possibility exists that a small number of deaths may have been overlooked, but based on the close monitoring of relocated animals in this case, this is unlikely.

It is also unlikely that the *Theileria* sp. (sable) is simply a mild strain (as is suggested for the *Theileria* sp. isolated in the research by Spitalska *et al.* 2005) and that clinical disease would thus not result in severe symptoms or death, since *Theileria* sp. (sable) infection was demonstrated to be the cause of death in various wildlife species by Nijhof *et al*. (2005). The *T. separata* infection, on the other hand, may well fall into this category, since it is traditionally known as a benign disease (Lawrence *et al*. 1994) in domestic animals (sheep). As speculated above, however, we may in fact be dealing with the same organism here, and since the *Theileria* spp. isolated are phylogenetically closer to *T. separata* than to *Theileria* sp. (sable), they may in fact be benign infections not capable of causing clinical disease. This would be especially true if this organism did in fact originate in wildlife since then one would expect the wildlife host to have developed tolerance to this pathogen over time.

Assuming these two *Theileria* are not the same spp. and that *T. separata* did not originate in wildlife, then it may be a simple case of the infected animals having developed immunity through previous exposure to these two pathogens. One needs to consider that in this case naïve animals may react very differently to infection from either, or both, *Theileria* species. This would require further investigation for any claims in this regard to be made.

Whatever the reason, we did not see clinical disease in this instance and it would thus appear that a situation of endemic stability exists here, as is the case with other protozoan infections such as *Babesia* infections in many species (Penzhorn, 2006).

Whether these *Theileria* spp. identified already existed in the area in other wildlife species, and the tsessebe were infected post arrival, or whether the *Theileria* spp. were introduced with the tsessebe when they were introduced into Vaalbos National Park, is another question we cannot answer without further investigation. Sampling of other species – both introduced and native to the area – would assist in this regard. This is an important consideration in terms of the endemic status of these organisms to this part of South Africa, not just to the tsessebe sampled.

Further investigation would also highlight the importance of these infections to other species. If the *Theileria* spp. isolated are more closely related to *Theileria* sp. (sable) on the basis of their biological characteristics, then they may be capable of causing serious disease and death in other species. One would assume that both the red hartebeest and possibly the blue wildebeest would be especially susceptible, based on their genetic close relatedness to the tsessebe (Arctander *et al.* 1999; Spitalska *et al.* 2005).

The implication of these two organisms on domestic species also has to be taken into consideration. *Theileria* sp. (sable) (AY748462) has not yet been isolated from domestic animals and thus its potential effect on domestic species is unknown and may warrant further investigation. However, a *Theileria* sp. very similar to *Theileria* sp. (sable) has recently been isolated from clinically ill dogs in South Africa, illustrating the importance of this consideration (Matjila, Leisewitz, Oosthuizen, Jongejan & Penzhorn, 2008). While *T. separata* is a known pathogen of sheep, it is considered a benign infection and should thus be of no significance.

If the *Theileria* spp. identified in this study result in (primarily) benign infections, under normal circumstances, then these organisms may have a very wide geographic distribution, potentially in many different host species. This is because the movement of both wildlife/antelope and domestic species has for many years taken place on a large scale worldwide without checks for the presence of *Theileria* spp. This sentiment is echoed by Chae *et al.* (1999). The only limiting factor would be the presence of a suitable tick vector to ensure continued transmission.

The tick species collected, *Rhipicephalus evertsi evertsi*, is known to occur in the Northern Cape Province where these samples were collected (Walker, Bouattour, Camicas, Estrada-Pena, Horak, Latif, Pegram & Preston, 2003) and is also known to be able to transmit *Theileria* spp. (*T. taurotragi* and *T. separata*: Lawrence *et al*. 1994; *T. equi*: Walker *et al.* 2003). This tick is widespread and common on livestock throughout much of Africa. Larvae and nymphs infest the same hosts as the adults (several domestic

species) and also infest scrub hares and various smaller antelope species. All stages infest a very wide variety of wild ungulates and other animals (Walker *et al.* 2003). Thus it is no surprise to find these ticks on the tsessebe. Based on this research, no deductions can be made with regard to the vector capability in transmitting *Theileria* sp. (sable) and *T. separata*, since no ticks were collected from any of the positive animals, and furthermore no DNA was isolated from the collected ticks for testing. However it is quite possible, and likely in my opinion, that this tick can act as a vector based on existing knowledge of its capacity as a vector for *T. separata* and the similarity of the *Theileria* spp. isolated here when compared to both *Theileria* sp. (sable) and *T. separata*. The vector capabilities of *Rhipicephalus evertsi evertsi* in this regard do, however, require further investigation to be absolutely sure of its ability to act as a vector for the isolated *Theileria* spp.

Low tick numbers were collected in this study since many animals had no or very few ticks on them, mostly because the capture operation was carried out in winter, and also because many animals could not be adequately restrained to collect blood and ticks.

Another potential reason, or added reason, for low numbers may be an innate resistance to tick infestation. Lightfoot and Norval (1981) reported that "as yet no tick problems have been encountered in square-lipped rhinoceros (*Ceratotherium simum*), warthog (*Phacochoerus aethiopicus*), wildebeest and tsessebe and it appears that these species have a high degree of innate resistance to tick infestation." Perhaps this is indeed the case and hence the reason why we see no significant cases of tick-borne disease in tsessebe, which would also help explain why so little research has been done in this regard to date. Obviously several other factors such as climatic conditions, prey density, etc., will also play a role in determining tick burdens, and hence also the opportunity for disease to be passed on to the host species.

In conclusion, since this was a random sampling and all animals appeared to be in good health, it is fair to assume that, as with several other wildlife species, the *Theileria* spp. identified do not cause disease under normal circumstances and that a situation of endemic stability exists. The significance of these infections may vary greatly once the

host is under any form of stress, however, and overt clinical disease may well be evident under such circumstances. This is especially true if the isolated *Theileria* spp. prove to be closer to *Theileria* sp. (sable) than to *T. separata*, based on biological characteristics, and in fact phylogenetically the isolated *Theileria* spp. do appear most closely related to *Theileria* sp. (sable) – figure 4.2. Further research will have to be done in order to better classify the isolated *Theileria* spp. in this regard, and in so doing determine their significance to both wildlife and domestic species.

Further investigation is required into potential tick vectors, but based on existing knowledge it is fair to assume that *Rhipicephalus evertsi evertsi*, the tick collected from the tsessebe, is capable of acting as a vector for the isolated *Theileria* spp.

In the interim the significance of these two parasites should not be underestimated, and care should be taken not to transmit the organisms into new areas (either through movement of infected animals showing no clinical signs, or through tick vectors capable of spreading these parasites), thus exposing naïve wildlife and domestic animals to the infection and perhaps causing clinical disease and losses, and the establishment of new *Theileria* spp. in areas they did not previously occur in.

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