Parasitology

cambridge.org/par

The host-specificity of *Theileria* sp. (sable) and *Theileria* sp. (sable-like) in African Bovidae and detection of novel *Theileria* in antelope and giraffe

Ronel Pienaar[1], Antoinette Josemans[1], Abdalla A. Latif[2] and Ben J. Mans[1,3,4]

[1]Epidemiology, Parasites and Vectors, Agricultural Research Council-Onderstepoort Veterinary Research, Onderstepoort 0110, South Africa; [2]School of Life Sciences, University of KwaZulu-Natal, Durban, Westville, South Africa; [3]The Department of Veterinary Tropical Diseases, University of Pretoria, Pretoria, South Africa and [4]Department of Life and Consumer Sciences, University of South Africa, Pretoria, South Africa

Research Article

Cite this article: Pienaar R, Josemans A, Latif AA, Mans BJ (2020). The host-specificity of *Theileria* sp. (sable) and *Theileria* sp. (sable-like) in African Bovidae and detection of novel *Theileria* in antelope and giraffe. *Parasitology* **147**, 213–224. https://doi.org/10.1017/S003118201900132X

Received: 14 May 2019
Revised: 18 July 2019
Accepted: 3 September 2019
First published online: 30 September 2019

Key words:
Bovidae; next-generation sequencing; real-time PCR; *Theileria* sp. (sable); *Theileria* sp. (sable-like)

Author for correspondence:
Ben J. Mans, E-mail: mansb@arc.agric.za

Abstract

Tick-borne diseases caused by *Theileria* are of economic importance in domestic and wildlife ruminants. The majority of *Theileria* infects a limited number of host species, supporting the concept of host specificity. However, some *Theileria* seem to be generalists challenging the host specificity paradigm, such as *Theileria* sp. (sable) reported from various vertebrate hosts, including African buffalo, cattle, dogs and different antelope species. We tested the hypothesis that *T.* sp. (sable) uses Bovidae as hosts in general using a real-time polymerase chain reaction assay specific for *T.* sp. (sable) and a closely related genotype: *T.* sp. (sable-like). Various antelope species from the Tragelaphini (black wildebeest, blesbuck, blue wildebeest, gemsbuck, sable and waterbuck) tested positive for either *T.* sp. (sable) or *T.* sp. (sable-like). However, no African buffalo ($n = 238$) or cattle ($n = 428$) sampled in the current study tested positive, suggesting that these latter species are not carrier hosts. The results were confirmed using next-generation sequencing which also indicated at least 13 new genotypes or species found in various antelope and giraffes. Genotypes were found in single host species or in evolutionarily related hosts, suggesting that host specificity in *Theileria* may be a lineage specific phenomenon likely associated with tick-host-parasite co-evolution.

Introduction

Theileria is tick-transmitted apicomplexan parasites of vertebrates that also serves as carrier hosts (Bishop *et al.*, 2004). Tick vector competence and geographic distribution, persistence in the host, as well as host specificity and host geographic distribution determine parasite prevalence (Mans *et al.*, 2015). The question of how host specific *Theileria* is, given incidental records for *Theileria* species in atypical hosts, remain (Mans *et al.*, 2015). Host specificity may impact on hypotheses for speciation, epidemiology, geographic distribution and clinical disease etiology.

Theileria sp. (sable) infection causes a high-mortality rate in antelope calves. The infections in these rare and endangered species constrain their translocation and animal breeding. The introduction of naive animals into endemic areas can result in high fatalities (Wilson *et al.*, 1974; Nijhof *et al.*, 2005; Steyl *et al.*, 2012). *Theileria* sp. (sable) and its disease have mostly been identified in roan and sable antelope.

Using reverse line blot (RLB) analysis, *T.* sp. (sable) has been detected in African buffalo (*Syncerus caffer*), cattle, blesbuck (*Damaliscus pygargus*), blue wildebeest (*Connochaetes taurinus*), klipspringer (*Oreotragus oreotragus*), reedbuck (*Redunca arundinum*), nyala (*Tragelaphus angasii*), sable antelope (*Hippotragus niger*), roan antelope (*Hippotragus equinus*) and dogs (Nijhof *et al.*, 2005; Matjila *et al.*, 2008; Muhanguzi *et al.*, 2010; Yusufmia *et al.*, 2010; Chaisi *et al.*, 2011; Pfitzer *et al.*, 2011; Adamu *et al.*, 2014; Eygelaar *et al.*, 2015; Njiiri *et al.*, 2015; Tembo *et al.*, 2018). It was also detected using sequencing in red hartebeest (*Alcelaphus buselaphus caama*) (Spitalska *et al.*, 2005). It, therefore, seems to be ubiquitous in a variety of host species and may be described as a host generalist parasite. However, cross reactivity on RLB has been noted between *T.* sp. (sable) and *T. velifera* (Brothers *et al.*, 2011; Mans *et al.*, 2011), while direct next-generation sequencing (NGS) did not detect *T.* sp. (sable) in cattle or buffalo (Mans *et al.*, 2016). Therefore, host specificity and correlations on co-infection with other genotypes remain uncertain (Njiiri *et al.*, 2015).

A closely related genotype, *T.* sp. (sable-like), was previously detected in a cattle individual with extreme signs of theileriosis, using conventional cloning and sequencing of the 18S rRNA gene (Mans *et al.*, 2011), but was not detected using NGS of larger buffalo and cattle populations (Mans *et al.*, 2016). The host of this genotype and its relationship to *T.* sp. (sable) remains obscure. In the current study, we investigated the host specificity of two *Theileria* genotypes with controversial host associations: *T.* sp. (sable) and *T.* sp. (sable-like). The prevalence of these two genotypes in various bovids was studied by using a novel real-time hybridization

CAMBRIDGE UNIVERSITY PRESS

assay capable of distinguishing these two genotypes. The study was conducted to specifically test the hypothesis that African buffalo and cattle are hosts for these genotypes, as reported previously using RLB analysis, where high prevalence ranging from 18–47% was observed (Muhanguzi *et al.*, 2010; Yusufmia *et al.*, 2010; Eygelaar *et al.*, 2015; Njiiri *et al.*, 2015; Tembo *et al.*, 2018). To validate the novel assay, the *Theileria* diversity in various antelopes was also investigated using a NGS approach previously used to study diversity in cattle and African buffalo (Mans *et al.*, 2016). The study also describes several novel genotypes from antelope and giraffe.

Materials and methods

Samples and DNA extraction

Blood samples ($n = 979$) submitted to the Agricultural Research Council – Epidemiology, Parasites and Vectors (ARC-EPV) diagnostic laboratory for routine *T. parva* testing were selected for analysis in this study. These included African buffalo ($n = 238$), black wildebeest (*Connochaetes gnou*) ($n = 16$), blesbuck ($n = 22$), blue wildebeest ($n = 20$), bushbuck (*Tragelaphus scriptus*) ($n = 4$), cattle (mixed breeds) ($n = 428$), eland (*Taurotragus oryx*) ($n = 10$), gemsbuck (*Oryx gazella*) ($n = 18$), giraffe (*Giraffa camelopardalis*) ($n = 31$), impala (*Aepyceros melampus*) ($n = 17$), kudus of the genus *Tragelaphus* ($n = 10$), nyala ($n = 14$), red hartebeest ($n = 8$), sable antelope ($n = 73$), sheep (*Ovis aries*) ($n = 29$) from Free State Province, springbok (*Antidorcas marsupialis*) ($n = 35$) and waterbuck (*Kobus ellipsiprymnus*) ($n = 6$). Cattle and antelope samples originated from the Corridor-disease endemic region in KwaZulu-Natal (Pienaar *et al.*, 2018). The sable antelope originated from Zambia. Buffalo were sampled from the Kruger National Park (KNP) or Hluhluwe-Imfolozi National Park (HIN) and cattle (mixed Nguni breeds) from diptanks adjacent to the parks. DNA was extracted from 200 μL blood and eluted in 100 μL elution buffer using automated MagNa Pure technology (Roche Diagnostics, Mannheim, Germany). Each polymerase chain reaction (PCR) reaction included 2.5 μL of DNA (~15–50 ng μL^{-1}).

Theileria sp. (sable) and *T.* sp. (sable-like) real-time PCR assay conditions

Hybridization assays for *T.* sp. (sable) and *T.* sp. (sable-like) were designed for use on the LightCycler® 480 (Roche Diagnostics, Mannheim, Germany). Each reaction consisted of 0.5 pmol of the *T.* sp. (sable) forward and reverse primers (TspSF: TGCATT GCCTTTTCTCCT TG, TspSR: CCTACTTTATTATTCCATGCT AA), 0.1 pmol of the *T.* sp. (sable) anchor (5′-GAGTTGATGCA TTGCGGCTTAT-FL) and probe (LC640-TCGGTCATGGTTTT CCTTG-PH) (Fig. 1), 1U uracil deoxy-glycosylase (UDG) (Roche Diagnostics, Mannheim, Germany) and 4 μL of the Hybrid PCR mix in a final volume of 20 μL. The Hybrid PCR mix consists of 2 μL each of the LightCycler® Fast Start DNA Master Plus and LightCycler® Genotyping Master mix (Roche Diagnostics, Mannheim, Germany) (Pienaar *et al.*, 2011*b*). Cycle conditions were started with 10 min UDG activation at 40 °C, followed by pre-incubation at 95 °C (10 min). An initial 10 cycles of denaturation at 95 °C (10 s), annealing at 57 °C (10 s) and extension at 72 °C (15 s) were followed by a 2-cycle touch-down of 5 °C in annealing temperature to 47 °C. This was followed by 34 cycles of denaturation at 95 °C (10 s), annealing at 47 °C (10 s) and extension at 72 °C (15 s). A positive and negative control was included in each run. Positive controls used was *T.* sp. (sable) originally isolated and propagated in culture from an infected roan antelope (*Hippotragus equinus*) (Zweygarth *et al.*, 2009), while for *T.* sp. (sable-like), the bovine field isolate (28 914) previously confirmed through sequencing and RLB analysis was used (Mans *et al.*, 2011). The negative control was from a bovine born and raised under quarantined tick-free conditions. Samples were also tested using the real-time PCR assays for *T. parva*, *T.* sp. (buffalo) and *T.* sp. (bougasvlei) as previously described (Pienaar *et al.*, 2011*a*, 2014).

Analytical sensitivity of the real-time PCR assays

A *T.* sp. (sable) and *T.* sp. (sable-like) 18S DNA template was prepared by a conventional PCR from the positive controls using the 18S primers from Allsopp *et al.* (1993) that yielded a ~1100 bp product. The PCR products were fractionated by agarose electrophoresis and the bands cut out before purification using Wizard® SV Gel and PCR Clean-Up System (Promega). The purified products were quantified spectrophotometrically using an ND-1000 NanoDrop spectrophotometer (NanoDrop Technologies, Inc) and concentrations confirmed by agarose electrophoresis against quantified size standards. A tenfold serial dilution was made from quantified 18S templates and tested in triplicate using the various assays to determine the analytical sensitivity. Crossing point (Cp) values were generated using the software methodology of the LightCycler for qualitative detection. The percentage efficiency of the PCR reactions was determined from the slopes of the regression lines of the Log [C]/Cp value plots using the formula, $\text{Efficiency} = 100(-1 + 10^{(-1/\text{slope})})$ according to Pfaffl (2004).

Specificity of the real-time PCR assays

Samples identified as positive for various *Theileria* genotypes using Sanger sequencing was used to confirm the analytical specificity of the *T.* sp. (sable) and *T.* sp. (sable-like) real-time PCR assays. The various genotypes tested are indicated in the Results section.

Next-generation sequencing of antelope samples

The procedures to sequence and analyse the 18S V4 hypervariable region using Roche GS-Junior NGS technology was followed as described (Mans *et al.*, 2016). This included amplification of the 18S V4 hypervariable region using universal RLB primers, tagging with sample specific identification tags, quality processing, using Basic Local Alignment Search Tool (BLAST) analysis (Altschul *et al.*, 1990) and pattern searching using electronic signatures to identify and count specific genotypes (Supplementary material). Unique genotypes were also confirmed using conventional ABI sequencing of the 18S gene as previously described, by sequencing 10 random clones from each sample (Mans *et al.*, 2011). Sequences were deposited in Genbank under the accession numbers MK131245–MK131258.

Bioinformatic analysis of the 18S rRNA V4 hypervariable region

Sequences were retrieved from Genbank using BLAST analysis (Altschul *et al.*, 1990), using the novel sequences to retrieve closely related sequences. A curated non-redundant *Theileria* sequence dataset (Mans *et al.*, 2015) was also included that represent the *Theileria sensu strictu* clade (Oosthuizen *et al.*, 2009). *Theileria equi* was used as an appropriate root for the tree since it generally groups outside the *Theileria sensu strictu* clade (Mans *et al.*, 2015). Sequences were aligned using multiple alignment using fast Fourier transform with parameters Q-INS-I that takes RNA secondary interaction into consideration and a 20PAM/k = 2 nucleotide scoring matrix (Katoh and Standley,

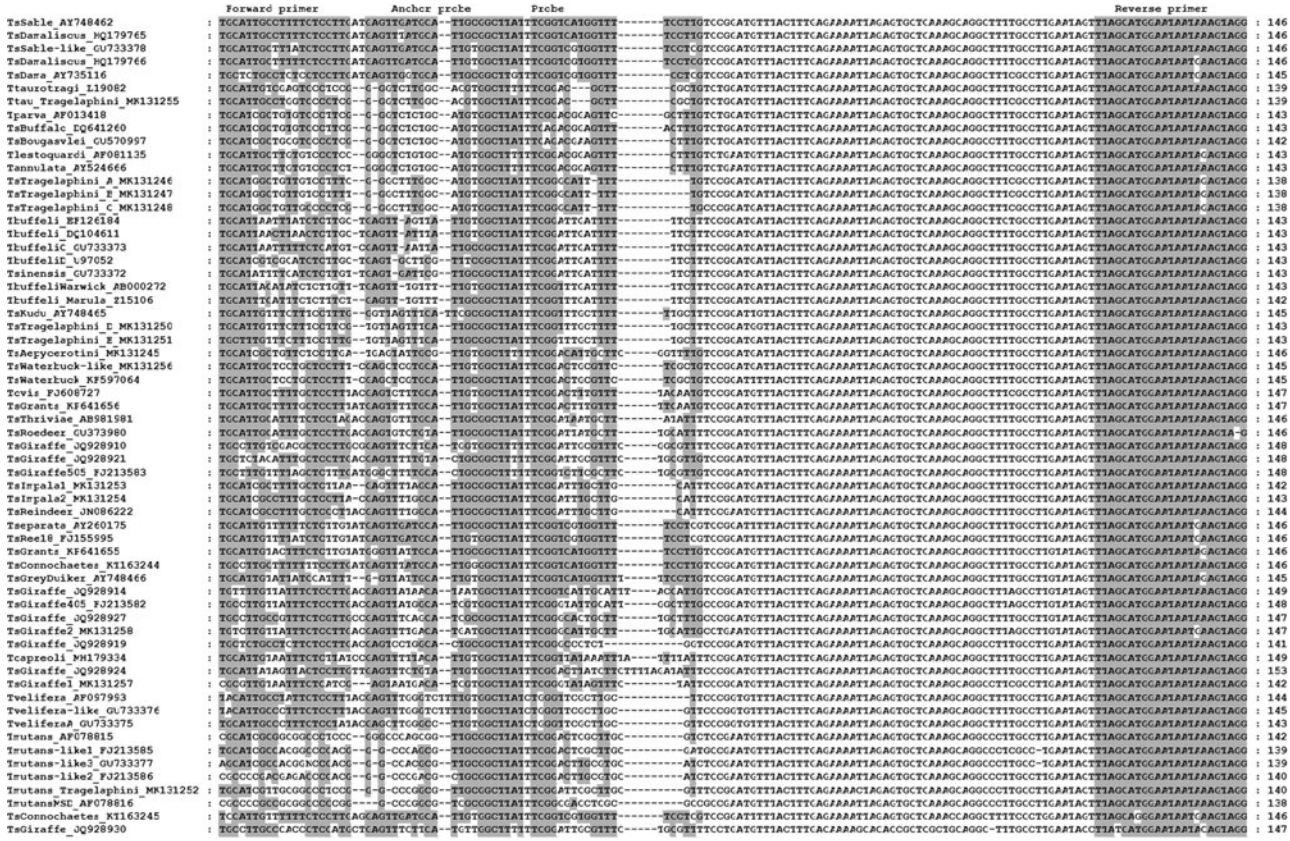

Fig. 1. Multiple sequence alignment of the 18S hypervariable region for various *Theileria* species. Conserved regions for the primer, anchor and donor sequences for *T.* sp. (sable) are shaded in grey.

2013). The alignment was trimmed to yield an alignment size of 264 bp that included the V4 hypervariable region. Phylogenetic analysis was performed with Mega 5 (Tamura *et al.*, 2011), using neighbour-joining with 10 000 bootstraps and the Kimura 2-parameter nucleotide substitution model. Uniform rates among sites and homogenous patterns among lineages were used and gaps or missing data were treated as partial deletion at 90% site coverage cutoff resulting in 224 sites used in the final analysis.

Results

Development of the T. *sp. (sable) and* T. *sp. (sable-like) real-time PCR assay*

Primers that specifically amplify both *T.* sp. (sable) and *T.* sp. (sable-like) were designed with a single set of anchor–donor hybridization probes (Fig. 1). The anchor–donor hybridization probes can differentiate both genotypes, but with different melting profiles and T_m values, for *T.* sp. (sable-like) at 50 ± 2 °C and *T.* sp. (sable) at 60 ± 2 °C (Fig. 2). During screening, another genotype was also detected that corresponded with *T.* sp. Ex. *Damaliscus lunatus* (HQ179765) (Brothers *et al.*, 2011), which gave a T_m at 58 ± 2 °C (Fig. 2). However, the difference in sequence of the V4 hypervariable region consists of only a single bp with *T.* sp. (sable) and we consider this a variant of the same genotype (Mans *et al.*, 2011). Conversely, *T.* sp. (sable) and *T.* sp. (sable-like) differ by four nucleotides in the V4 hypervariable region, suggesting they are different species (Mans *et al.*, 2011, 2015). *Theileria* sp. (sable-like) also differs from *T.* sp. Ex. *Damaliscus lunatus* (HQ179766) (Brothers *et al.*, 2011), with one nucleotide difference outside the probe area. We consider this to be a variant of the same genotype as well, based on the arguments of Mans *et al.* (2011). The latter showed that single nucleotide polymorphisms occur in the V4 hypervariable region for *T. parva*, while established species always differ by greater than three nucleotide differences in the V4 hypervariable region. One to three differences in the V4 hypervariable region may therefore be indicative of variation within a species.

Analytical sensitivity of the real-time PCR assays

Analytical sensitivity was determined using ten-fold serial dilutions of a quantified 18S rRNA template. Both assays had similar sensitivity capable of detecting up to ~10 molecules per reaction with an efficiency of 85–88% (Fig. 3). For each assay a cut-off was instated at 37 cycles.

Specificity of the assay

The assay only detected *T.* sp. (sable) and *T.* sp. (sable-like) genotypes and did not detect any genotypes found in cattle and buffalo including *T. annulata* (AY524666), *T. buffeli* (Warwick) (AB000272), *T. cf. buffeli* C (GU733373), *T. lestoquardi* (AF081135), *T. mutans* (AF078815), *T. mutans*-like 1 (FJ213585), *T. mutans*-like 2 (FJ213586), *T. mutans*-like 3 (GU733377), *T. mutans* MSD (AF078816), *T. parva* (AF013418), *T. taurotragi* (L19082), *T.* sp. (buffalo) (DQ641260), *T.* sp. (bougasvlei) (GU570997), *T. velifera* (AF097993), *T. velifera* A (GU733375) and *T. velifera* B (GU733376) (Fig. 2). In addition, 22 genotypes unique to antelope identified in samples from the current study using NGS were also not detected (Fig. 2). This included *T. mutans*-like (Tragelaphini) (MK131252), *T. ovis* (FJ608727), *T. separata* (AY260175), *T.* sp. (Aepycerotini) (MK131245), *T.* sp. (giraffe) 1 (MK131257), *T.* sp. (giraffe) 2 (MK131258), *T.* sp. (giraffe) 405 (FJ213582), *T.* sp. (giraffe) 505 (FJ213583), *T.* sp. (giraffe) (JQ928914), *T.* sp. (giraffe) (JQ928927), *T.* sp. (impala) Cervidae-like 1 (MK131253), *T.* sp. (impala) Cervidae-like 2

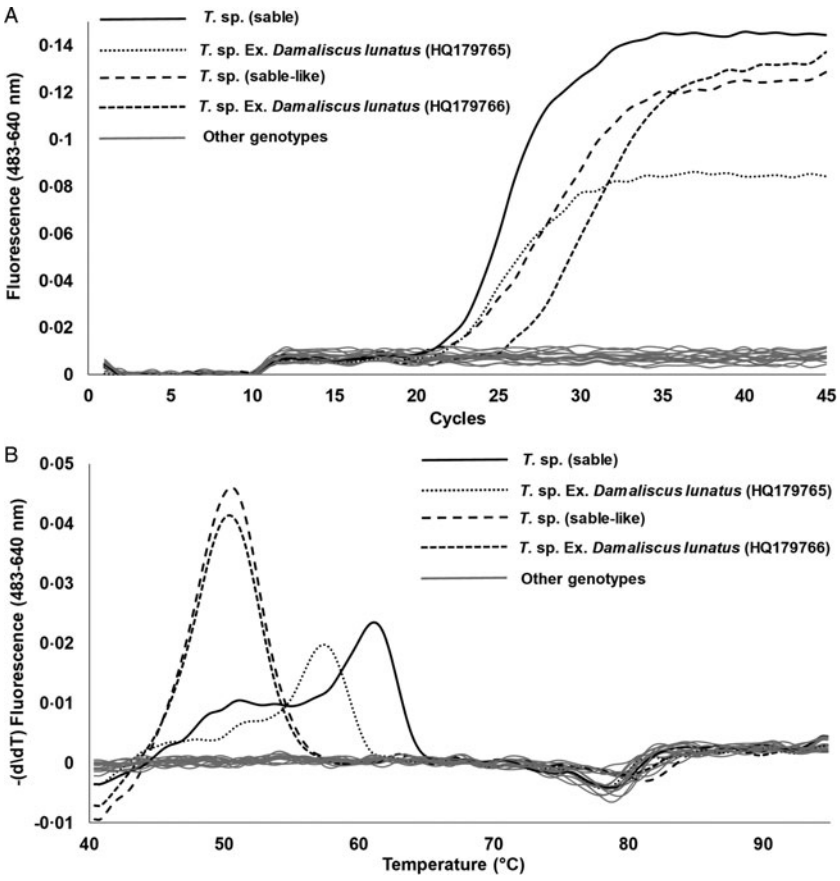

Fig. 2. The hybridization assay for *T.* sp. (sable) and *T.* sp. (sable-like). (A) Amplification profiles for the positive controls and other genotypes tested in the study (section 'Specificity of the assay'). (B) Melting curves for the positive controls and other genotypes tested in the study (section 'Specificity of the assay').

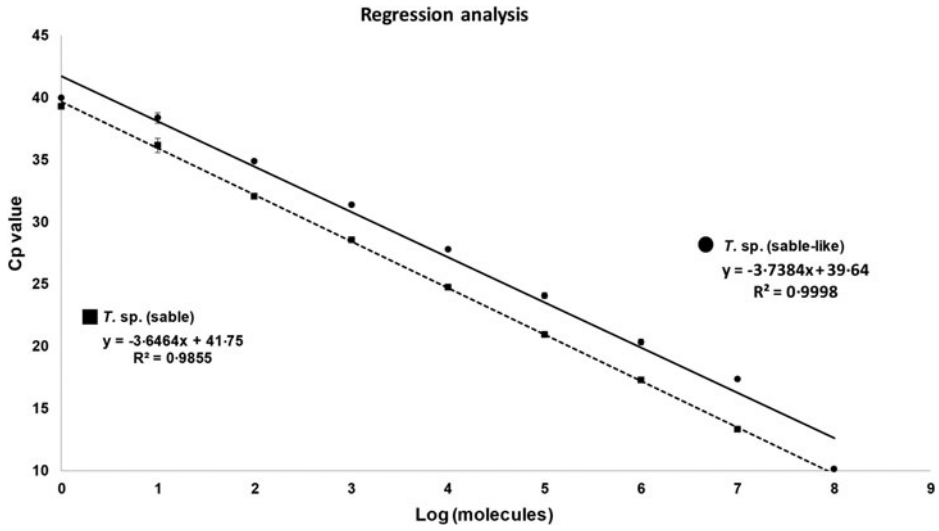

Fig. 3. Sensitivity of the *T.* sp. (sable) and *T.* sp. (sable-like) assays. A ten-fold dilution series were performed in triplicate using a quantified 18S DNA template for each genotype. Indicated regression analysis of Cp values for triplicate samples and the equation for the regression analysis, its R^2 value and the efficiency calculated from the slope. S.D. values are indicated by error bars.

(MK131254), *T.* sp. (kudu) (AY748465), *T.* sp. Ree (FJ155995), *T.* sp. (Tragelaphini) A (MK131246), *T.* sp. (Tragelaphini) B (MK131247), *T.* sp. (Tragelaphini) C (MK131248), *T.* sp. (Tragelaphini) D (MK131250), *T.* sp. (Tragelaphini) E (MK131251), *T.* sp. (waterbuck) (KF597064), *T.* sp. (waterbuck-like) (MK131256) and *T. taurotragi*-like (Tragelaphini) (MK131255).

Real-time PCR assay results for antelope, buffalo, cattle and sheep

A variety of animals that included antelope, buffalo, cattle and sheep were screened using the *T.* sp. (sable) and *T.* sp. (sable-like) assays (Table 1). Animals positive for *T.* sp. (sable) included black wildebeest, blue wildebeest, gemsbuck, sable and sheep, while animals positive for *T.* sp. (sable-like) included black wildebeest, blesbuck, blue wildebeest, sable and waterbuck. Neither buffalo nor cattle were positive for *T.* sp. (sable) or *T.* sp. (sable-like). Conversely, none of the antelope tested were positive for *T. parva*, *T.* sp. (buffalo) or *T.* sp. (bougasvlei) (results not shown).

Relative parasitaemia of T. *sp. (sable) and* T. *sp. (sable-like)*

Frequency distribution curves of the crossing-point values indicate that for both *T.* sp. (sable) and *T.* sp. (sable-like), a normal distribution is observed that range from 22–34 cycles (Fig. 4). Within this range, the Cp value distribution is similar for different antelope species, suggesting that the parasitaemia range for these

Table 1. Summary of results for *T.* sp. (sable) and *T.* sp. (sable-like) obtained from the real-time hybridization assay

	T. sp. (sable)				*T.* sp. (sable-like)			
Animal	POS	NEG	Total	Percentage	POS	NEG	Total	Percentage
Buffalo	0	238	238	0	0	238	238	0
Cattle	0	428	428	0	0	428	428	0
Black wildebeest	12	4	16	75	10	6	16	63
Blesbuck	7	15	22	32	13	9	22	59
Blue wildebeest	18	2	20	90	12	8	20	60
Bushbuck	0	4	4	0	0	4	4	0
Eland	0	10	10	0	0	10	10	0
Gemsbuck	14	4	18	78	0	18	18	0
Giraffe	0	31	31	0	0	31	31	0
Impala	0	17	17	0	0	17	17	0
Kudu	0	10	10	0	0	10	10	0
Nyala	0	14	14	0	0	14	14	0
Redhartbeest	0	8	8	0	0	8	8	0
Sable	9	64	73	12	35	38	73	48
Sheep	16	13	29	55	0	29	29	0
Springbuck	0	35	35	0	0	35	35	0
Waterbuck	0	6	6	0	6	0	6	100

species lies well within the detection range of the real-time PCR assays. As such, the majority of field carrier animals should be detectable.

Validation of the T. *sp. (sable) and* T. *sp. (sable-like) assays using next-generation sequencing*

Previously, the *Theileria* diversity in cattle and buffalo was validated using conventional and NGS approaches, to allow confidence when assessing specificity of the *T. parva*, *T.* sp. (buffalo), *T.* sp. (bougasvlei) and *T. taurotragi* real-time PCR assays (Mans *et al.*, 2011, 2016; Pienaar *et al.*, 2011*a*, 2011*b*, 2014, 2018). In the case of antelopes, a large-scale diversity assessment has never been performed, making the assessment of potential false positive detection difficult when implementing new assays. To address this and assist in the estimation of specificity of the *T.* sp. (sable) and *T.* sp. (sable-like) assays, the V4 hypervariable region of the 18S rRNA gene from various antelopes was sequenced using a NGS approach.

The majority of common genotypes found in cattle or buffalo were not found in any antelope using NGS. This included *T. parva*, *T.* sp. (buffalo), *T.* sp. (bougasvlei), *T. mutans* 1, *T. mutans* 2, *T. mutans* 3, *T. mutans*, *T. mutans* MSD, *T. buffeli* Warwick, *T. buffeli* C, *T. velifera* and *T. velifera* B. Genotypes previously detected in cattle or buffalo were *T. taurotragi* and *T. velifera* A. *Theileria taurotragi* was found in eland and kudu (Fig. 5). *Theileria velifera* A was found in eland, kudu and nyala (Fig. 5).

NGS confirmed the positive status of those samples detected with the hybridization assay, while no *T.* sp. (sable) or *T.* sp. (sable-like) sequences were detected in samples that tested negative for these genotypes using the real-time PCR assays (Table 2). Conversely, thirteen novel genotypes not previously published were detected in various antelopes and confirmed using conventional sequencing (Fig. 5; Table 3). This included novel genotypes found in bushbuck, eland, giraffe, impala, kudu, nyala and sable, namely *T.* sp. (Aepycerotini) (MK131245), *T.* sp. (giraffe) 1A (MK131257), *T.* sp. (giraffe) 2A (MK131258), *T.* sp. (impala) Cervidae-like 1 (MK131253), *T.* sp. (impala) Cervidae-like 2 (MK131254), *T. mutans*-like (Tragelaphini) (MK131252), *T.* sp. (Tragelaphini) A (MK131246), *T.* sp. (Tragelaphini) B (MK131247), *T.* sp. (Tragelaphini) C (MK131248), *T.* sp. (Tragelaphini) D (MK131250), *T.* sp. (Tragelaphini) E (MK131251), *T.* sp. (waterbuck-like) (MK131256) and *T. taurotragi*-like (Tragelaphini) (MK131255).

Known genotypes were also found that included *T. ovis* (FJ608727), *T. separata* (AY260175), *T.* sp. (giraffe) 405 (FJ213582), *T.* sp. (giraffe) 505 (FJ213583), *T.* sp. (giraffe) (JQ928914), *T.* sp. (giraffe) (JQ928927), *T.* sp. (kudu) (AY748465), *T.* sp. Ree (FJ155995), *T.* sp. (waterbuck) (KF597064) in blesbuck, black wildebeest, blue wildebeest, eland, gemsbuck, giraffe, kudu, sable, sheep, springbuck and waterbuck. The majority of antelope species were infected with more than one *Theileria* species or genotype.

Discussion

Accurate diagnostics and epidemiological data depend on sensitive and specific assays to be of any practical or scientific utility (Mans *et al.*, 2015). Accurate quantitative real-time hybridization PCR assays have been developed for *T. parva*, *T.* sp. (buffalo) and *T.* sp. (bougasvlei) (Sibeko *et al.*, 2008; Pienaar *et al.*, 2011*b*, 2014). Hybridization probe assays can differentiate genotypes based on differences in anchor and probe regions (Mans *et al.*, 2011). The current study describes the development of a sensitive and specific real-time hybridization probe assay capable of differentiating *T.* sp. (sable) and *T.* sp. (sable-like).

The analytical sensitivity of the assays is comparable to other *Theileria* real-time PCR assays (Sibeko *et al.*, 2008; Papli *et al.*, 2011; Pienaar *et al.*, 2011*b*, 2014, 2018). The frequency distribution of the Cp values follows normal distributions with parasitaemia levels in carrier hosts spanning detectable ranges of the assay. Ranges observed are similar to *T. parva*, *T.* sp. (buffalo), *T.* sp. (bougasvlei) and *T. taurotragi* (Pienaar *et al.*, 2011*b*, 2014, 2018). Parasitaemia levels range from 0.001–0.1% and

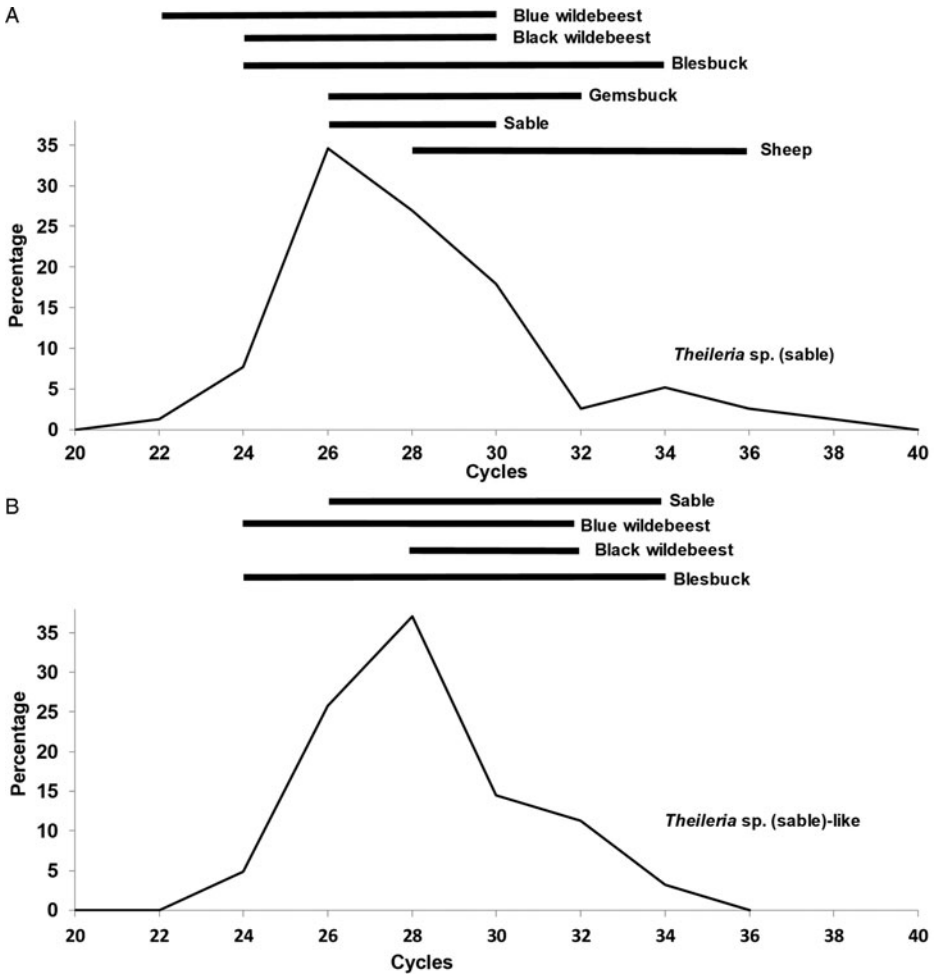

Fig. 4. Frequency distribution plots of the Cp values for (A) *T.* sp. (sable) and (B) *T.* sp. (sable-like). Horizontal bars indicate the distribution range found in different hosts that covers >80% of the distribution values for that host.

seem to be the norm for the carrier state in *Theileria sensu strictu* (Pienaar *et al.*, 2011*a*, 2011*b*).

The assays for *T.* sp. (sable) and *T.* sp. (sable-like) detected no positive cattle ($n = 428$) or buffalo ($n = 238$), while antelope showed prevalence's of 12–90% for much smaller sample sizes. Prevalence estimates ranged from 18–47% for buffalo and cattle using RLB analysis (Muhanguzi *et al.*, 2010; Yusufmia *et al.*, 2010; Eygelaar *et al.*, 2015; Njiiri *et al.*, 2015; Tembo *et al.*, 2018). The presence of *T.* sp. (sable) in cattle and buffalo reported using RLB is therefore probably erroneous (Mans *et al.*, 2011, 2016).

The current study highlights a pitfall of RLB analysis, i.e. cross-hybridization may lead to erroneous detection of species. The probe for *T. velifera* differs in three nucleotides from *T.* sp. (sable) suggesting this similarity level may result in cross-hybridization under non-stringent hybridization conditions. Cross-hybridization may not be restricted to *T.* sp. (sable) and *T. velifera*, since the probe for *T. mutans* differs with 2–3 nucleotides for members of the *T. mutans* clade and cross-reactivity was observed for members (*T. mutans*-like 1, *T. mutans*-like 2 and *T. mutans*-like 3) exclusive to African buffalo (Mans *et al.*, 2011, 2016). Cross-reactivity may also occur with *T. mutans*-like (Tragelaphini) identified in bushbuck. The probe for *T. buffeli* is identical for the majority of clade members including *T. buffeli* C and *T. sinensis*-like that seem to be specific for African buffalo (Mans *et al.*, 2011, 2016). Since many genotypes show host specificity, RLB analysis could lead to under- or overestimation of prevalence reducing the impact of epidemiological studies (Mans *et al.*, 2016). Other drawbacks of RLB such as PCR competition for the universal primers may lead to suppression of low-abundance templates and underestimation of genotypes since the majority of hosts are infected by multiple species (Mans *et al.*, 2011, 2016), PCR suppression is a major factor limiting the use of RLB for epidemiological studies. The classic example is *T. parva* in African buffalo, where 27–64% infection was detected using RLB, while real-time PCR assays detected ~70% positive samples (Pienaar *et al.*, 2011*a*). Alternatives to RLB would be species-specific real-time PCR assays (Mans *et al.*, 2015). Conversely, RLB has been successful in detecting novel genotypes when present as single infections but still needs sequencing for identification and confirmation (Nijhof *et al.*, 2003, 2005; Oosthuizen *et al.*, 2008, 2009; Chaisi *et al.*, 2013, 2014). An alternative to this would be a direct sequencing approach as described in the current and previous studies (Mans *et al.*, 2011, 2016). While NGS may replace real-time PCR applications in the long term (Mans *et al.*, 2016), the latter remains cheaper and faster for routine diagnostics making species-specific PCR assays the current preferred choice within a diagnostic setting.

NGS of antelope confirmed that the primers and probes for the *T.* sp. (sable) and *T.* sp. (sable-like) hybridization assays are specific, while also allowing discovery of a number of unique genotypes specific to antelopes. A number of novel genotypes were exclusive to the Tragelaphini (bushbuck, eland, kudu and nyala) suggesting that these are unique species that may infect the Tragelaphini in general. *Theileria* sp. (kudu) (AY748465) was found in the greater kudu (Nijhof *et al.*, 2005) and the current study found this genotype in eland, suggesting that Tragelaphini may be general hosts. *Theileria* cf. *velifera* A was shown to be prevalent in the Tragelaphini while extensive screening showed its presence in cattle but not in African buffalo (Mans *et al.*, 2011, 2016). Conversely, the related genotypes *T. velifera* and *T. velifera* B were extensively detected in cattle and African buffalo

Fig. 5. Phylogenetic analysis of the *Theileria sensu strictu* clade. Indicated are various *Theileria* genotypes or species, their Genbank accession numbers in brackets and the number of animals in which *Theileria* were detected for various host species using next-generation or Sanger sequencing. The neighbour-joining tree was constructed using Mega 5 (Tamura *et al*., 2011). *Theileria equi* was used to root the tree. Nodal support is for 10 000 bootstraps and only support above 50% is shown.

(Mans *et al*., 2016), but not in any wild antelope from the current study.

With regard to host specificity in antelope, it was indicated that *T. taurotragi* infect the Tragelaphini, possibly due to evolution of this species in the last common ancestor, with only a more recent adaptation to cattle (Pienaar *et al*., 2018). It was suggested that Tragelaphini in general may be hosts for *T. taurotragi* (Pienaar *et al*., 2018) and was confirmed for the mountain bongo (*Tragelaphus eurycerus isaaci*) (Bishop *et al*., 2019). NGS of the wildlife samples confirmed this again.

The discovery of the related novel *T.* sp. *taurotragi*-like genotype is of interest since it was unique to bushbuck, a member of the Tragelaphini, suggesting speciation due to geographic isolation of bushbuck. It is of interest that bushbuck in the current study was negative for *T. taurotragi*, but positive for *T.* sp. *taurotragi*-like, although bushbuck was found to be carriers of *T. taurotragi* in Uganda (Oura *et al*., 2011). Genetic incompatibility has been suggested as a mechanism for speciation in *Theileria* as observed for *T.* sp. (buffalo) and *T.* sp. (bougasvlei) (Pienaar *et al*., 2014), and may operate here as well, but will need a larger sampling of bushbuck to confirm this. Mitochondrial analysis indicated that bushbuck may consist of different species in Central and South Africa (Hassanin *et al*., 2012), so that host specificity may also play a role in this instance. Alternatively, the possibility exists that the bushbuck from Uganda was carriers of *T.* sp. *taurotragi*-like and not *T. taurotragi*, since these genotypes differ

Table 2. Summary of results for *T. sp.* (sable) and *T.* sp. (sable-like) obtained from NGS.

	T. sp. (sable)				*T.* sp. (sable-like)			
Animal	POS	NEG	Total	Percentage	POS	NEG	Total	Percentage
Buffalo[a]	0	672	672	0	0	672	672	0
Cattle[a]	0	478	478	0	0	478	478	0
Black wildebeest	11	5	16	69	12	4	16	75
Blesbuck	7	12	19	37	13	6	19	68
Blue wildebeest	7	0	7	100	6	1	7	86
Bushbuck	0	4	4	0	0	4	4	0
Eland	0	10	10	0	0	10	10	0
Gemsbuck	10	1	11	91	0	11	11	0
Giraffe	0	27	27	0	0	27	27	0
Impala	0	15	15	0	0	15	15	0
Kudu	0	10	10	0	0	10	10	0
Nyala	0	13	13	0	0	13	13	0
Red hartebeest	0	8	8	0	0	8	8	0
Sable	2	46	48	4	24	24	48	50
Springbuck	0	35	35	0	0	35	35	0
Waterbuck	0	6	6	0	6	0	6	100

Indicated are the number of positive and negative animals for each animal species tested.
[a]Results collated from Mans *et al.* (2016).

by one nucleotide in the RLB probe area and would probably show cross-reactivity. In this case, host specificity will exist within the Tragelaphini for *T. taurotragi* and related genotypes. An important question is whether the hydrolysis probe assay for *T. taurotragi* (Pienaar *et al.*, 2018), would also detect *T.* sp. *taurotragi*-like, since these genotypes differ by one nucleotide in the hydrolysis probe region. However, the *T. taurotragi* forward primer differs in three positions towards the 3′end of the primer and linked with the touchdown PCR conditions employed ensure specificity, since none of the bushbuck samples tested positive with this assay (Pienaar *et al.*, 2018). Piroplasms previously observed in bushbuck from South Africa were named *Theileria tragelaphi* (Neitz, 1931; Bigalke *et al.*, 1972). The current study indicated at least four different genotypes associated with bushbuck that would obscure the identity of the original named piroplasm species.

Antelope infected by *T.* sp. (sable) and *T.* sp. (sable-like) includes Alcelaphini (blesbuck, blue wildebeest, black wildebeest, red hartebeest and tsessebe) and Hippotragini (gemsbuck and sable antelope) which form a monophyletic clade. They also form a larger monophyletic group with the Caprini (goats and sheep) (Hassanin *et al.*, 2012), suggesting that these species may also be infected by *T.* sp. (sable) and *T.* sp. (sable-like). Screening of 29 sheep indicated 55% infected with *T.* sp. (sable) and was confirmed for two sheep using conventional Sanger sequencing. *Theileria* sp. (sable) was also reported for sheep in South Africa using RLB (Berggoetz *et al.*, 2014). Extensive screening of small ruminants (chamois, sheep and goats) from the northern hemisphere did not report *T.* sp. (sable) (Altay *et al.*, 2007; García-Sanmartín *et al.*, 2007; Torina *et al.*, 2007; Iqbal *et al.*, 2013; Aydin *et al.*, 2015; Ozubek and Aktas, 2017; Chaligiannis *et al.*, 2018). This is likely due to *Rhipicephalus appendiculatus* and *Rhipicephalus evertsi evertsi*, considered tick vectors for *T.* sp. (sable) (Steyl *et al.*, 2012), only occurring in sub-Saharan Africa (Walker *et al.*, 2000). The Alcelaphini, Hippotragini and Caprini with the Oreotragini (klipspringer), Cephalotragini (duiker), Reduncini (waterbuck, Leche, Reedbuck), Antelopini (Gazelle and Springbuck), Neotragini (Suni, Royal antelope) and Aepycerotini (Impala) form the Antilopinae (Hassanin *et al.*, 2012). The waterbuck screened in the current study showed the presence of *T.* sp. (sable-like). *Theileria* sp. (sable) was also previously detected in klipspringer and reedbuck (Nijhof *et al.*, 2005). All Antilopinae may therefore be potential hosts. The original description of *T.* sp. (sable-like) in a single cattle sample seemed to have been incidental, since it has not been found in other cattle sampled to date (Mans *et al.*, 2011, 2016), but has now been extensively found in various antelope species.

Additional *Theileria* genotypes detected in the Antilopinae included *T. ovis*, *T.* sp. Ree, *T.* sp. waterbuck, *T. separata* and *T.* sp. waterbuck-like (sable). These genotypes do not seem to be host specific within the Antilopinae (Hassanin *et al.*, 2012).

The only genotypes that fall outside this picture are specific for impala, including *T.* sp. (Aepycerotini), *T.* sp. (Impala) Cervidae-like 1 and *T.* sp. (Impala) Cervidae-like 2. Impala did not present any of the genotypes found in other Antilopinae. The impala (with the Neotragini) group basals to all other Antilopinae (Hassanin *et al.*, 2012). Their relationship to the Antilopinae has been considered uncertain leading to the elevation of its own evolutionary lineage, Aepycerotinae (Ansell, 1971; Vrba, 1979; Gentry, 1992; Matthee and Davis, 2001). Their unique makeup of *Theileria* genotypes would support this. An unnamed *Theileria* species serologically distinct from *Theileria* isolated from Bovidae in East Africa, not infective to cattle but infective to impala by piroplasm inoculation was described (Grootenhuis *et al.*, 1975). Using RLB analysis *T. bicornis*, *T. buffeli* and *T.* sp. (sable) were detected in impala from South Africa (Berggoetz *et al.*, 2014). While these species may also infect impala, the current study suggests that further investigation need to confirm this.

The *Theileria* sp. (Aepycerotini) group with no defined clade, while *T.* sp. (Impala) Cervidae-like 1 and *T.* sp. (Impala) Cervidae-like 2 groups within a well-supported clade with *T.* sp. (reindeer), a genotype related to *Theileria* from North Texas

Table 3. Sequencing results for NGS and Sanger sequencing

Tribe	Antelope (NGS/Sanger)	Genotype	NGS	Sanger
Alcelaphini	Blesbuck (19/2)	Tovis_FJ608727	12	0
		Tsable_AY748462	11	1
		TspSable_like_GU733378	11	1
Alcelaphini	Blue wildebeest (7/4)	Tovis_FJ608727	7	2
		Tsable_AY748462	4	4
		TspSable_like_GU733378	6	3
Alcelaphini	Black wildebeest (15/2)	Tsable_AY748462	11	1
		TspSable_like_GU733378	12	1
Hippotragini	Gemsbuck (11/3)	TspRee18_FJ155995	10	2
		Tsable_AY748462	10	3
Hippotragini	Sable (48/5)	Tovis_FJ608727	31	4
		Tseparata_AY260175	6	2
		Tsable_AY748462	2	0
		TspSable_like_GU733378	25	2
		TspWaterbuck-like_MK131256	44	3
Caprini	Sheep (0/2)	Tsable_AY748462	NA	2
Reduncini	Waterbuck (6/2)	Tsp_waterbuck_KF597072	6	1
		TspSable_like_GU733378	6	2
Antelopini	Springbuck (8/1)	Tseparata_AY260175	4	1
		TspRee18_FJ155995	6	1
Aepycerotini	Impala (15/2)	TspAepycerotini_MK131245	15	3
		TspImpala_Cervidae-like1_MK131253	11	2
		TspImpala_Cervidae-like2_MK131254	11	2
Tragelaphini	Bushbuck (4)	TspTragelaphini_B_MK131247	4	1
		TspTragelaphini_D_MK131250	4	1
		Tmut-like_Tragelaphini_MK131252	2	0
		Ttau-like_Tragelaphini_MK131255	4	2
Tragelaphini	Eland (7/3)	TspTragelaphini_A_MK131246	3	1
		TspTragelaphini_B_MK131247	0	1
		TspTragelaphini_E_MK131251	6	6
		Tmut-like_Tragelaphini_MK131252	6	1
		TspKudu_AY748465	6	1
		TcfveliferaA_GU733375	7	2
		Ttau_L19082	2	2
Tragelaphini	Kudu (10/2)	TspTragelaphini_A_MK131246	5	1
		TspTragelaphini_D_MK131250	1	0
		TspTragelaphini_E_MK131251	10	1
		Tmut-like_Tragelaphini_MK131252	8	2
		TspKudu_AY748465	10	1
		TcfveliferaA_GU733375	8	2
		Ttau_L19082	6	0
Tragelaphini	Nyala (13/2)	TspTragelaphini_B_MK131247	2	0
		TspTragelaphini_C_MK131248	3	1
		TspTragelaphini_D_MK131250	13	2
		TspTragelaphini_E_MK131251	1	0
		Tmut-like_Tragelaphini_MK131252	3	0

(*Continued*)

Table 3. (*Continued.*)

Tribe	Antelope (NGS/Sanger)	Genotype	NGS	Sanger
		TcfveliferaA_GU733375	12	2
Giraffidae	Giraffe (28/3)	TspGiraffe0405_FJ213582	26	3
		TspGiraffe0505_FJ213583	8	2
		TspGiraffe1A_MK131257	24	3
		TGiraffe2A_MK131258	16	1
		TspGiraffe_NG2012b_JQ928927	15	0
		TspGiraffe_NG-2012b_JQ928914	17	2

Indicated are the number of animals that was positive using either NGS or Sanger sequencing. Genotypes shows an abbreviated name with its Genbank accession number.

white-tail deer (Garner *et al.*, 2012). Reindeer and white-tail deer belong to the Odocoileini (Cervidae) and are genetically distant from Bovidae (Hassanin *et al.*, 2012). The high sequence similarity observed between *Theileria* from Cervidae and impala is of interest, suggesting that the presence of these parasites (either in impala or reindeer/white-tail deer) was due to a recent introduction into Africa or into North America. It is tempting to speculate that impala is the ancestral host since their origin could be dated to ~16–18 MYA, while the Odocoileini (reindeer/white-tail deer) has originated only ~5.7 MYA (Hassanin *et al.*, 2012). However, the diversity of *Theileria* genotypes in antelope from various continents and their evolutionary origins still needs elucidation.

The current study identified numerous genotypes described in giraffe from South Africa and Kenya (Oosthuizen *et al.*, 2009; Githaka *et al.*, 2013). In addition, two novel genotypes (*T.* sp. giraffe 1 and *T.* sp. giraffe 2) were detected, the former identical to a giraffe genotype sampled from a zoo in China, originating from South Africa (Zhang *et al.*, 2016). As such, *Theileria* diversity in giraffes from southern Africa is as extensive as in East Africa (Githaka *et al.*, 2013).

Lineage specificity seems to emerge as a theme for *Babesia* and *Theileria* species, suggesting co-evolution of parasite and host with origins in the last common host ancestors. This may enable dating of the origin of parasite lineages using fossil and molecular clock records for various antelopes (Hassanin *et al.*, 2012). For *T.* sp. (sable) and *T.* sp. (sable-like) this implies that they originated ~16–12 MYA in the ancestral lineage of the Alcelaphini, Caprini, Hippotragini and Reduncini (Hassanin *et al.*, 2012). This is similar to the origin estimated for the *T. taurotragi* clade that occurred after divergence of the Bovini and Tragelaphini (Pienaar *et al.*, 2018).

In conclusion, the current study indicated host specificity for *T.* sp. (sable) and *T.* sp. (sable-like) within the Tragelaphini, with no conclusive evidence of infection of African buffalo or cattle. NGS indicates that *Theileria* genotypes are specific to either antelope or bovines, even though host specificity may be for lineages and not necessarily individual species. While a variety of novel *Theileria* genotypes have been detected, their tick vectors remain unknown, impacting on a full understanding of their epidemiology, geographical ranges and veterinary significance.

Financial support. The research project was funded and approved by the Department of Agriculture, Forestry and Fisheries (DAFF) (Project number: P10000062).

Conflict of interest. None.

Ethical standards. Not applicable.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S003118201900132X.

References

Adamu M, Troskie M, Oshadu DO, Malatji DP, Penzhorn BL and Matjila PT (2014) Occurrence of tick-transmitted pathogens in dogs in Jos, Plateau State, Nigeria. *Parasites & Vectors* **7**, 119.

Allsopp BA, Baylis HA, Allsopp MTPE, Cavalier-Smith T, Bishop RP, Carrington DM, Sohanpal B and Spooner P (1993) Discrimination between six species of *Theileria* using oligonucleotide probes which detect small subunit ribosomal RNA sequences. *Parasitology* **107**, 157–165.

Altay K, Dumanli N and Aktas M (2007) Molecular identification, genetic diversity and distribution of *Theileria* and *Babesia* species infecting small ruminants. *Veterinary Parasitology* **147**, 161–165.

Altschul SF, Gish W, Miller W, Myers EW and Lipman DJ (1990) Basic local alignment search tool. *Journal of Molecular Biology* **215**, 403–410.

Ansell WFH (1971) Order artiodactyla. In Meester J and Setzer HW (eds), *The Mammals of Africa: an Identification Manual*. Washington, DC: Smithsonian Institution Press, pp. 15–83.

Aydin MF, Aktas M and Dumanli N (2015) Molecular identification of *Theileria* and *Babesia* in ticks collected from sheep and goats in the Black Sea region of Turkey. *Parasitology Research* **114**, 65–69.

Berggoetz M, Schmid M, Ston D, Wyss V, Chevillon C, Pretorius AM and Gern L (2014) Tick-borne pathogens in the blood of wild and domestic ungulates in South Africa: interplay of game and livestock. *Ticks and Tick Borne Diseases* **5**, 166–175.

Bigalke RD, Keep ME and Schoeman JH (1972) Some protozoan parasites of tragelaphine antelopes in South Africa with special reference to a *Babesia* sp. in a bushbuck and a *Trypanosoma theileri*-like parasite in a nyala. *Onderstepoort Journal of Veterinary Research* **39**, 225–228.

Bishop R, Musoke A, Morzaria S, Gardner M and Nene V (2004) *Theileria*: intracellular protozoan parasites of wild and domestic ruminants transmitted by ixodid ticks. *Parasitology* **129**, S271–S283.

Bishop RP, Odongo DO, Dolan TT, Dolan RB, Skilton RA and Sayer PD (2019) Theileriosis in mountain bongo repatriated to Kenya: a clinical and molecular investigation. *Journal of Zoo and Wildlife Medicine* **50**, 342–349.

Brothers PS, Collins NE, Oosthuizen MC, Bhoora R, Troskie M and Penzhorn BL (2011) Occurrence of blood-borne tick-transmitted parasites in common tsessebe (*Damaliscus lunatus*) antelope in Northern Cape Province, South Africa. *Veterinary Parasitology* **183**, 160–165.

Chaisi ME, Sibeko KP, Collins NE, Potgieter FT and Oosthuizen MC (2011) Identification of *Theileria parva* and *Theileria* sp. (buffalo) 18S rRNA gene sequence variants in the African Buffalo (*Syncerus caffer*) in Southern Africa. *Veterinary Parasitology* **182**, 150–162.

Chaisi ME, Janssens ME, Vermeiren L, Oosthuizen MC, Collins NE and Geysen D (2013) Evaluation of a real-time PCR test for the detection and discrimination of *Theileria* species in the African buffalo (*Syncerus caffer*). *PLoS ONE* **8**, e75827.

Chaisi ME, Collins NE and Oosthuizen MC (2014) Phylogeny of *Theileria buffeli* genotypes identified in the South African buffalo (*Syncerus caffer*) population. *Veterinary Parasitology* **204**, 87–95.

Chaligiannis I, Fernández de Mera IG, Papa A, Sotiraki S and de la Fuente J (2018) Molecular identification of tick-borne pathogens in ticks collected from dogs and small ruminants from Greece. *Experimental and Applied Acarology* **74**, 443–453.

Eygelaar D, Jori F, Mokopasetso M, Sibeko KP, Collins NE, Vorster I, Troskie M and Oosthuizen MC (2015) Tick-borne haemoparasites in African buffalo (*Syncerus caffer*) from two wildlife areas in Northern Botswana. *Parasites & Vectors* **8**, 26.

García-Sanmartín J, Aurtenetxe O, Barral M, Marco I, Lavin S, García-Pérez AL and Hurtado A (2007) Molecular detection and characterization of piroplasms infecting cervids and chamois in Northern Spain. *Parasitology* **134**, 391–398.

Garner BC, Holman P and Berent LM (2012) Theileriosis in a reindeer (*Rangifer tarandus tarandus*) associated with a potentially novel *Theileria* sp. *Veterinary Clinical Pathology* **41**, 497–501.

Gentry AW (1992) The subfamilies and tribes of the family Bovidae. *Mammal Review* **22**, 1–32.

Githaka N, Konnai S, Skilton R, Kariuki E, Kanduma E, Murata S and Ohashi K (2013) Genotypic variations in field isolates of *Theileria* species infecting giraffes (*Giraffa camelopardalis* tippelskirchi and *Giraffa camelopardalis reticulata*) in Kenya. *Parasitology International* **62**, 448–453.

Grootenhuis JG, Young AS, Kimber CD and Drevemo SA (1975) Investigations on a *Theileria* species from an impala. *Journal of Wildlife Diseases* **11**, 122–127.

Hassanin A, Delsuc F, Ropiquet A, Hammere C, Jansen van Vuuren B, Matthee C, Ruiz-Garcia M, Catzefli F, Areskoug V, Thanh Nguyen T and Couloux A (2012) Pattern and timing of diversification of Cetartiodactyla (Mammalia, Laurasiatheria), as revealed by a comprehensive analysis of mitochondrial genomes. *Comptes Rendus Biologies* **335**, 32–50.

Iqbal F, Khattak R, Ozubek S, Khattak M, Rasul A and Aktas M (2013) Application of the reverse line blot assay for the molecular detection of *Theileria* and *Babesia* sp. in sheep and goat blood samples from Pakistan. *Iran Journal of Parasitology* **8**, 289–295.

Katoh K and Standley DM (2013) MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Molecular Biology and Evolution* **30**, 772–780.

Mans BJ, Pienaar R, Latif AA and Potgieter FT (2011) Diversity in the 18S SSU rRNA V4 hyper-variable region of *Theileria* in bovines and African buffalo (*Syncerus caffer*) from Southern Africa. *Parasitology* **138**, 766–779.

Mans BJ, Pienaar R and Latif AA (2015) A review of *Theileria* diagnostics and epidemiology. *International Journal of Parasitology: Parasites and Wildlife* **4**, 104–118.

Mans BJ, Pienaar R, Ratabane J, Pule B and Latif AA (2016) Investigating the diversity of the 18S SSU rRNA hyper-variable region of *Theileria* in cattle and Cape buffalo (*Syncerus caffer*) from Southern Africa using a next generation sequencing approach. *Ticks and Tick-borne Diseases* **7**, 869–879.

Matjila PT, Leisewitz AL, Oosthuizen MC, Jongejan F and Penzhorn BL (2008) Detection of a *Theileria* species in dogs in South Africa. *Veterinary Parasitology* **157**, 34–40.

Matthee CA and Davis SK (2001) Molecular insights into the evolution of the family Bovidae: a nuclear DNA perspective. *Molecular Biology and Evolution* **18**, 1220–1230.

Muhanguzi D, Matovu E and Waiswa C (2010) Prevalence and characterization of *Theileria* and *Babesia* species in cattle under different husbandry systems in Western Uganda. *International Journal of Animal Veterinary Advances* **2**, 51–58.

Neitz WO (1931) Blood parasites of game in Zululand. Preliminary report. 17th Report of the Director of Veterinary Service and Animal Industry, Union of South Africa, pp. 45–60.

Nijhof AM, Penzhorn BL, Lynen G, Mollel JO, Morkel P, Bekker CP and Jongejan F (2003) *Babesia bicornis* sp. nov. and *Theileria bicornis* sp. nov.: tick-borne parasites associated with mortality in the black rhinoceros (*Diceros bicornis*). *Journal of Clinical Microbiology* **41**, 2249–2254.

Nijhof AM, Pillay V, Steyl J, Prozesky L, Stoltsz WH, Lawrence JA, Penzhorn BL and Jongejan F (2005) Molecular characterization of *Theileria* species associated with mortality in four species of African antelopes. *Journal of Clinical Microbiology* **43**, 5907–5911.

Njiiri NE, Bronsvoort BM, Collins NE, Steyn HC, Troskie M, Vorster I, Thumbi SM, Sibeko KP, Jennings A, van Wyk IC, Mbole-Kariuki M, Kiara H, Poole EJ, Hanotte O, Coetzer K, Oosthuizen MC, Woolhouse M and Toye P (2015) The epidemiology of tick-borne haemoparasites as determined by the reverse line blot hybridization assay in an intensively studied cohort of calves in western Kenya. *Veterinary Parasitology* **210**, 69–76.

Oosthuizen MC, Zweygarth E, Collins NE, Troskie M and Penzhorn BL (2008) Identification of a novel *Babesia* sp. from a sable antelope (*Hippotragus niger* Harris, 1838). *Journal of Clinical Microbiology* **46**, 2247–2251.

Oosthuizen MC, Allsopp BA, Troskie M, Collins NE and Penzhorn BL (2009) Identification of novel *Babesia* and *Theileria* species in South African giraffe (*Giraffa camelopardalis*, Linnaeus, 1758) and roan antelope (*Hippotragus equinus*, Desmarest 1804). *Veterinary Parasitology* **163**, 39–46.

Oura CA, Tait A, Asiimwe B, Lubega GW and Weir W (2011) *Theileria parva* genetic diversity and haemoparasite prevalence in cattle and wildlife in and around Lake Mburo National Park in Uganda. *Parasitology Research* **108**, 1365–1374.

Ozubek S and Aktas M (2017) Molecular and parasitological survey of ovine piroplasmosis, including the first report of *Theileria annulata* (Apicomplexa: Theileridae) in sheep and goats from Turkey. *Journal of Medical Entomology* **54**, 212–220.

Papli N, Landt O, Fleischer C, Koekemoer JO, Mans BJ, Pienaar R, Josemans A, Zweygarth E, Potgieter F and Latif AA (2011) Evaluation of a TaqMan real-time PCR for the detection of *Theileria parva* in buffalo and cattle. *Veterinary Parasitology* **175**, 356–359.

Pfaffl MW (2004) Quantification strategies in real-time PCR. In Bustin SA (ed.), *A-Z of Quantitative PCR*. La Jolla, CA, USA: International University Line (IUL), pp. 87–112.

Pfitzer S, Oosthuizen MC, Bosman AM, Vorster I and Penzhorn BL (2011) Tick-borne blood parasites in nyala (*Tragelaphus angasii* Gray 1849) from KwaZulu-Natal, South Africa. *Veterinary Parasitology* **176**, 126–131.

Pienaar R, Potgieter FT, Latif AA, Thekisoe OMM and Mans BJ (2011a) Mixed *Theileria* infections in free-ranging buffalo herds: implications for diagnosing *Theileria parva* infections in Cape buffalo (*Syncerus caffer*). *Parasitology* **138**, 884–895.

Pienaar R, Potgieter FT, Latif AA, Thekisoe OMM and Mans BJ (2011b) The Hybrid II assay: a sensitive and specific real-time hybridization assay for the diagnosis of *Theileria parva* infection in Cape buffalo (*Syncerus caffer*) and cattle. *Parasitology* **138**, 884–895.

Pienaar R, Potgieter FT, Latif AA, Thekisoe OMM and Mans BJ (2014) Geographic distribution of *Theileria* sp. (buffalo) and *Theileria* sp. (bougasvlei) in Cape buffalo (*Syncerus caffer*) in Southern Africa: implications for speciation. *Parasitology* **141**, 411–424.

Pienaar R, Latif AA and Mans BJ (2018) Investigations into the host specificity of *Theileria taurotragi*. *Veterinary Parasitology* **254**, 30–35.

Sibeko KP, Oosthuizen MC, Collins NE, Geysen D, Rambritch NE, Latif AA, Groeneveld HT, Potgieter FT and Coetzer JA (2008) Development and evaluation of a real-time polymerase chain reaction test for the detection of *Theileria parva* infections in Cape buffalo (*Syncerus caffer*) and cattle. *Veterinary Parasitology* **155**, 37–48.

Spitalska E, Riddell M, Heyne H and Sparagano OA (2005) Prevalence of theileriosis in Red Hartebeest (*Alcelaphus buselaphus caama*) in Namibia. *Parasitology Research* **97**, 77–79.

Steyl JC, Prozesky L, Stoltsz WH and Lawrence JA (2012) Theileriosis (Cytauxzoonosis) in Roan antelope (*Hippotragus equinus*): field exposure to infection and identification of potential vectors. *Onderstepoort Journal of Veterinary Research* **79**, E1–E8.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M and Kumar S (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution* **28**, 2731–2739.

Tembo S, Collins NE, Sibeko-Matjila KP, Troskie M, Vorster I, Byaruhanga C and Oosthuizen MC (2018) Occurrence of tick-borne haemoparasites in cattle in the Mungwi District, Northern Province, Zambia. *Ticks and Tick Borne Diseases* **9**, 707–717.

Torina A, Vicente J, Alongi A, Scimeca S, Turlá R, Nicosia S, Di Marco V, Caracappa S and de la Fuente J (2007) Observed prevalence of tick-borne pathogens in domestic animals in Sicily, Italy during 2003–2005. *Zoonoses and Public Health* **54**, 8–15.

Vrba ES (1979) Phylogenetic analysis and classification of fossil and recent Alcelaphini Mammalia: Bovidae. *Biological Journal of the Linnean Society* **11**, 207–228.

Walker JB, Keirans JE and Horak IG (2000) *The Genus Rhipicephalus (Acari, Ixodidae): a Guide to the Brown Ticks of the World*. Cambridge: Cambridge University Press.

Wilson DE, Bartsch RC, Bigalke RD and Thomas SE (1974) Observations on mortality rates and disease in roan and sable antelope on nature reserves in the Transvaal. *South African Journal of Wildlife Research* **4**, 203–206.

Yusufmia SB, Collins NE, Nkuna R, Troskie M, Van den Bossche P and Penzhorn BL (2010) Occurrence of *Theileria parva* and other haemoprotozoa in cattle at the edge of Hluhluwe-iMfolozi Park KwaZulu-Natal, South Africa. *Journal of the South African Veterinary Association* **81**, 45–49.

Zhang Y, Li T, Cui Y, Wang J, Lv Y, Wang R, Jian F, Zhang L, Wang J, Yang G and Ning C (2016) The first report of *Anaplasma phagocytophilum* and a novel *Theileria* spp. co-infection in a South African giraffe. *Parasitology International* **65**, 347–351.

Zweygarth E, Benade J, Steyl J, Prozesky L, Koekemoer O and Josemans AI (2009) *In vitro* cultivation of a *Theileria* species from a roan antelope (*Hippotragus equinus*). *Parasitology Research* **105**, 1755–1757.