

## Occurrence of *Theileria parva* and other haemoprotozoa in cattle at the edge of Hluhluwe-iMfolozi Park, KwaZulu-Natal, South Africa

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### ABSTRACT

*Theileria parva*, the most important bovine theilerial species in sub-Saharan Africa, causes widespread mortality and morbidity in endemic areas. A survey was conducted using buffy-coat specimens from 60 apparently healthy adult communally herded Nguni-type cattle at the northeastern edge of the Hluhluwe-iMfolozi Park to determine, by means of PCR and Reverse Line Blot (RLB) hybridisation, the occurrence of *Theileria* and *Babesia* species. The presence of *Trypanosoma* species was determined using PCR-RFLP. Results showed that 6.7 % of the specimens were positive for *Theileria parva*. This significant finding suggests that cattle in South Africa, and not only African buffaloes (*Syncerus caffer*), may be subclinical carriers of *T. parva*. Other species identified were *T. mutans* (83.3 %), *T. velifera* (70.0 %), *Theileria* sp. (sable) (46.8 %) and *T. taurotragi* (1.7 %). Two specimens (3.3 %) were positive for *Babesia bovis* and single specimens (1.7 %) positive for *B. bigemina* and *B. rossi*, respectively. Mixed infections, of up to 4 species, were common (65.0 %). Only 1 specimen was found to be positive for *Trypanosoma vivax*, and 2 for *T. theileri*, of which only the first species is pathogenic.

**Keywords:** *Babesia bigemina*, *Babesia bovis*, *Babesia rossi*, cattle, Hluhluwe-iMfolozi Park, *Theileria mutans*, *Theileria parva*, *Theileria taurotragi*, *Theileria velifera*, *Theileria* sp. (sable), *Trypanosoma theileri*, *Trypanosoma vivax*, wildlife–livestock interface.

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### INTRODUCTION

Theileriosis, a tick-transmitted protozoan disease, is a major constraint for cattle production in the tropics and subtropics<sup>12</sup>. *Theileria parva* is the most important species in sub-Saharan Africa, while *T. annulata* occurs further northwards and eastwards. Three subspecies of *T. parva* were previously recognised: *T. parva parva*, causing classical East Coast fever (ECF)<sup>16</sup>; *T. parva laurencei*, causing Corridor disease<sup>17</sup>; and *T. parva bovis*, causing Zimbabwe theileriosis or January disease<sup>15</sup>. This subdivision has been abandoned<sup>34</sup>. East Coast fever is an acute, usually fatal, disease that is transmitted between infected cattle and susceptible cattle by the 3-host ixodid tick, *Rhipicephalus appendiculatus*<sup>16</sup>. Corridor disease is an acute disease transmitted principally from asymptomatic carrier African buffalo (*Syncerus caffer*)

to susceptible cattle by *R. appendiculatus* as well as by *R. zambeziensis* and possibly by *R. duttoni*<sup>17</sup>. Zimbabwe theileriosis is usually a milder form of the disease, with transmission by *R. appendiculatus* between infected and susceptible cattle<sup>15</sup>.

ECF was introduced to South Africa during 1901–1903 by cattle imported from Kenya and Tanzania for restocking after the ravages of rinderpest and the Anglo-Boer War<sup>14</sup>. It is estimated that 1.25 million of the 4 million cattle in the affected area had died of ECF by 1914. The disease was eradicated from southern Africa during a 50-year campaign consisting of movement control, tick control, destocking of infected pastures and slaughtering of infected herds<sup>16</sup>. The vector was not eradicated, however, and the original buffalo-associated *T. parva* remained endemic in South African and Zimbabwean buffalo populations<sup>40</sup>.

Corridor disease is an acute, usually fatal disease of cattle resembling ECF<sup>17</sup>. It is caused by infection with *T. parva* strains from African buffaloes, the only wild ruminant species that is a carrier of the causative organism<sup>36</sup>. The first outbreak in

South Africa occurred in the corridor between the then Hluhluwe and Umfolozi Game Reserves in KwaZulu-Natal (now incorporated in the Hluhluwe-iMfolozi Park), shortly after ECF had been eradicated<sup>27</sup>. The disease occurs sporadically wherever cattle are in contact with infected African buffaloes and in the presence of the vector ticks. In areas where cattle are raised, acceptance of buffaloes for conservation or recreation is a major constraint because of Corridor disease<sup>17</sup>. Buffalo-derived *T. parva* is universally distributed in wild buffaloes in southern Africa, except in the Addo Elephant National Park and some other game reserves in non-endemic areas where *T. parva*-free animals have been translocated<sup>30</sup>. In contrast to the situation in buffaloes, erythrocytic piroplasms in cattle are usually absent or too scanty to infect ticks, so the disease is usually self-limiting in cattle. The distinct seasonality of *R. appendiculatus*, which has a 2-year life cycle in southern Africa<sup>38</sup>, and transstadial transmission of *T. parva* also play a major role in this regard. The possibility of *T. parva* from buffalo transforming under natural conditions in South Africa to *T. parva* causing ECF has always been a highly contentious topic, as it places the movement of buffaloes under severe constraints<sup>36</sup>.

In South Africa, the Kruger National Park and Hluhluwe-iMfolozi Park are regarded as endemic areas for buffalo-derived *T. parva*. The South African Animal Disease Act (Act 35 of 1984) stipulates the following controlled veterinary acts to be performed in outbreaks of Corridor disease: *Susceptible animals*: Contact between cattle and African buffaloes shall be prevented; all cattle in a controlled area shall be dipped or sprayed regularly by the responsible person with an efficient remedy; no animal shall be chemotherapeutically treated without the written authorisation of the director. *Contact animals* shall be isolated and dipped or sprayed with an efficient remedy under the supervision of an officer or an authorised person in the manner and at the intervals determined by the responsible state veterinarian. *Infected animals* shall be

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isolated and dipped or sprayed with an efficient remedy under the supervision of an officer or an authorised person in the manner and at the intervals determined by the responsible state veterinarian. In contrast to the regulations pertaining to ECF, slaughter of infected animals is therefore not enforced, on the assumption that infected animals, which may not be treated, will succumb to the disease and will not become subclinical carriers.

The campaign to control the ECF epidemic in South Africa by intensive regulatory control of *R. appendiculatus* had a major effect on single-host ticks, including *Rhipicephalus (Boophilus) spp.*, ensuring that bovine babesiosis remained of secondary importance for many decades. In the latter part of the 20th century, relaxation or breakdown of tick-control measures and the advent of acaricide resistance in *Rhipicephalus (Boophilus) spp.*, caused bovine babesiosis to increase in prevalence and significance to the point where it became recognised as one of the most important livestock diseases in South Africa<sup>7,8</sup>.

Trypanosomosis is a complex, debilitating and often fatal disease caused by infection with one or more of the pathogenic tsetse fly-transmitted protozoan parasites of the genus *Trypanosoma*<sup>5</sup>. The most important species responsible for the disease complex, commonly known as 'nagana' in livestock, include *Trypanosoma brucei*, *T. congolense* and *T. vivax*<sup>1</sup>. Two tsetse fly species, *Glossina austeni* and *G. brevipalpis*, persisted in KwaZulu-Natal despite eradication attempts<sup>13</sup>.

A number of molecular, biochemical and immunological tools are available that enable differentiation between *Theileria* species<sup>4</sup>. Early molecular detection techniques involved the use of probes to detect repetitive regions in parasite genomic deoxyribonucleic acid (DNA). The polymerase chain reaction (PCR) assay has largely superseded DNA probes. PCR has a higher sensitivity and specificity for detecting parasite DNA in blood. False positives may occur, however, due to contamination. As it is not always possible to detect mixed infections with PCR, a reverse line blot (RLB) hybridisation assay targeting the 18S ribosomal ribonucleic acid (rRNA) gene has been developed for simultaneous identification and differentiation of distinct piroplasm species present in the same sample<sup>11</sup>. The RLB has proven to be a very powerful tool in detecting subclinical infections. It also gives an indication of new species or genotypes possibly being present in the sample<sup>32</sup>.

Bovine trypanosomosis has been shown to be prevalent in anaemic cattle in the

Table 1: Species-specific probes for *Theileria* and *Babesia* species.

Species	Probe sequence (from 5'-3')*
<i>B. felis</i>	TTA TGC GTT TTC CGA CTG GC
<i>B. divergens</i>	ACT RAT GTC GAG ATT GCA C
<i>B. microti</i>	GRC TTG GCA TCW TCT GGA
<i>B. bigemina</i>	CGT TTT TTC CCT TTT GTT GG
<i>B. bovis</i>	CAG GTT TCG CCT GTA TAA TTG AG
<i>B. rossi</i>	CGG TTT GTT GCC TTT GTG
<i>B. canis canis</i>	TGC GTT GAC CGT TTG AC
<i>B. canis vogeli</i>	AGC GTG TTC GAG TTT GCC
<i>B. major</i>	TCC GAC TTT GGT TGG TGT
<i>B. bicornis</i>	TTG GTA AAT CGC CTT GGT C
<i>B. caballi</i>	GTT GCG TTK TTC TTG CTT TT
<i>Theileria/Babesia catchall</i>	TAA TGG TTA ATA GGA RCR GTT G
<i>T. parva</i>	GGA CGG AGT TCG CTT TG
<i>T. annulata</i>	CCT CTG GGG TCT GTG CA
<i>T. mutans</i>	CTT GCG TCT CCG AAT GTT
<i>T. taurotragi</i>	TCT TGG CAC GTG GCT TTT
<i>T. velifera</i>	CCT ATT CTC CTT TAC GAG T
<i>T. lestoquardi</i>	CTT GTG TCC CTC CGG G
<i>T. equi</i>	TTC GTT GAC TGC GYT TGG
<i>T. buffeli</i>	GGC TTA TTT CGG WTT GAT TTT
<i>T. bicornis</i>	GCG TTG TGG CTT TTT TCT G
<i>Theileria</i> sp. (buffalo)	CAG ACG GAG TTT ACT TTG T
<i>Theileria</i> sp. (sable)	GCT GCA TTG CCT TTT CTC C
<i>Theileria</i> sp. (kudu)	CTG CAT TGT TTC TTT CCT TTG

\*Symbols used to indicate degenerate positions: R = A/G, W = A/T, K = G/T.

vicinity of the Hluhluwe-iMfolozi Park<sup>41</sup>. In a subsequent survey, blood specimens were taken randomly from healthy cattle in the same area to determine the occurrence of *Trypanosoma* species. This offered the opportunity to determine the occurrence of other haemoparasites, especially *Theileria parva*, in these cattle. The RLB was used for detection of *Theileria* species in these buffy-coat specimens and PCR-RFLP was used to determine occurrence of *Trypanosoma* species.

## MATERIALS AND METHODS

### Specimen collection

Specimens were collected during March 2006 from 60 randomly selected, adult, apparently healthy, communally-grazed Nguni-type cattle from various herds adjacent to the northeastern edge of the Hluhluwe-iMfolozi Park. The total number of cattle in the area was not known. Jugular blood was collected in Vacutainer<sup>®</sup> tubes coated with ethylenediamine-tetra-acetic acid (EDTA) as anticoagulant. Micro-capillary tubes were filled with blood from the Vacutainer tubes and centrifuged for 5 min at 9000 r.p.m. A diamond-tipped pen was used to cut the capillary tubes immediately above the buffy coat, and the buffy coat was extruded onto filter paper (Whatman no. 3). The buffy-coat-spotted filter paper was dried, sealed in a plastic bag containing silica gel and stored at

-20 °C until processed further at the Molecular Biology Laboratory, Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria.

### DNA extraction

DNA was extracted from 60 buffy coat filter papers (at least 3 disks, 3 mm in diameter per sample) using QIAamp DNA Mini kit (QIAGEN<sup>®</sup>), according to the manufacturer's instructions. DNA was eluted in 150 µl of AE buffer and stored at 4 °C.

### Polymerase Chain Reaction (PCR) for *Theileria* and *Babesia* species

Primers RLB F2 (5'-GAC ACA GGG AGG TAG TGA CAA G-3') and biotin-labeled RLB R2 (5'-Biotin-CTA AGA ATT TCA CCT CTA ACA GT-3') were used for amplification of the V4 hypervariable region of *Theileria* and *Babesia* 18S rRNA genes<sup>11</sup>. Reactions were performed in a final volume of 25 µl with Platinum Quantitative PCR Super mix-UDG (Invitrogen), 0.25 µl of each primer (20 pmol) and 2.5 µl of purified DNA. A touchdown PCR programme was conducted in the Gene Amp PCR system 9700. The cycling conditions were as follows: 3 min at 37 °C; 10 min at 94 °C; and 10 cycles of 94 °C for 20 s, 67 °C for 30 s, 72 °C for 30 s, with decreasing annealing temperature after every second cycle by 2 °C for 5 times until the annealing temperature reached 57 °C.

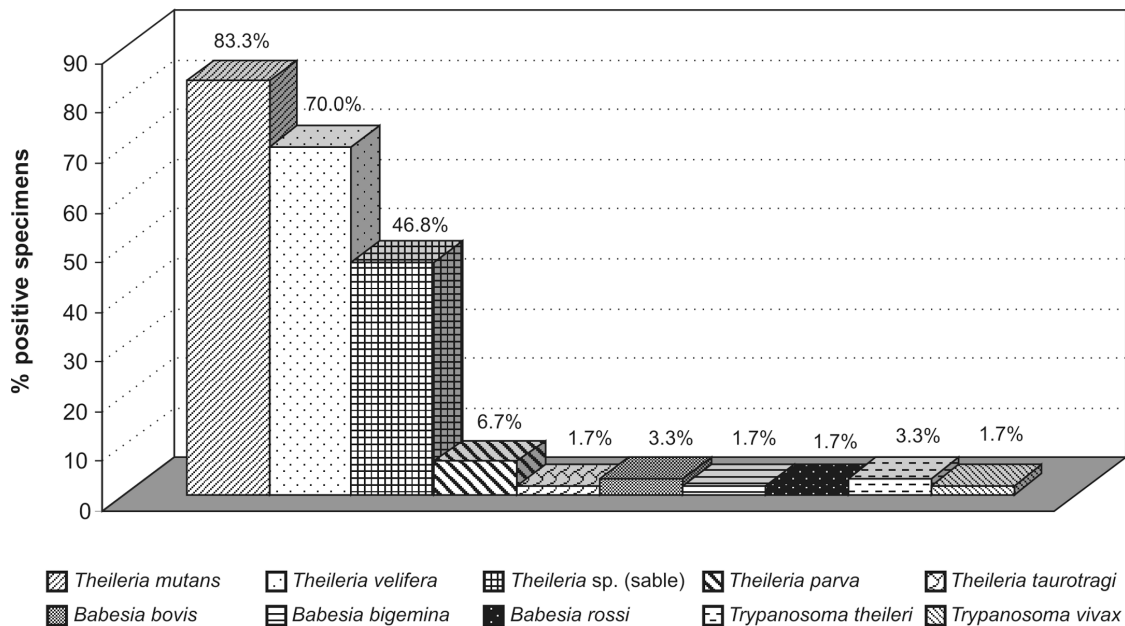


Fig. 1: Occurrence of specific haemoparasites in cattle along the northeastern edge of Hluhluwe-iMfolozi Park, KwaZulu-Natal, South Africa.

Finally 40 cycles of 94 °C for 20 s, 57 °C for 30 s and 72 °C for 30 s were performed. *Babesia bovis* was used as positive control, while bovine DNA was used as negative control.

#### Reverse Line Blot (RLB) hybridisation

The PCR products were analysed using the RLB hybridisation technique<sup>11,28,29</sup>. A *Theileria* and *Babesia* genus-specific oligonucleotide probe and 23 species-specific probes (Table 1) were included on the membrane.

#### PCR for *Trypanosoma* species

The primers used for the amplification of *Trypanosoma* species were as described<sup>10</sup>. The first amplification was done using the forward primer 18ST nF2 (5'-CAA CGA TGA CAC CCA TGA ATT GGG GA-3') and 18ST nR3 (5'-TGC GCG ACC AAT AAT TGC AAT AC-3') as reverse primer. A semi-nested second amplification was done using 18ST nF2 with the reverse primer 18ST nR2 (5'GTG TCT TGT TCT CAC TGA CAT TGT AGT G-3').

#### RFLP for differentiation of *Trypanosoma* species

The RFLP analysis of the 18S rRNA PCR products was performed<sup>10</sup>. Briefly, a 6 µl aliquot of amplified DNA was digested with *Msp1* and *Eco571* restriction enzymes in buffer Y+Tango with S-adenosyl-methionine according to the manufacturer's specifications (Gibco, UK) using 0.6 U of each enzyme per µl of PCR product in 15 µl total volume. The digestion was incubated overnight in a water bath at 37 °C. Restriction products were resolved on a 10 % polyacrylamide gel together with a 100 bp DNA ladder (MBI

Fermentas, Lithuania) for fragment size determination. DNA fragments were separated by horizontal electrophoresis in 0.5× TBE buffer at 100 V for 2.5 h. The gel was stained using Sybr Green I nucleic acid gel stain (Roche diagnostics)<sup>10</sup>.

#### RESULTS

Haemoprotozoa DNA could be extracted from all specimens. Occurrence of *Theileria* spp., *Babesia* spp. and *Trypanosoma* spp. is summarised in Fig. 1. *Theileria parva* occurred in 4 specimens (6.7%). *Theileria mutans* (83.3%) and *T. velifera* (70.0%) were the most prevalent *Theileria* species. These were also the only haemoparasites occurring as single-species infections: 10 specimens (16.7%) were infected with *T. mutans* only, and 5 specimens (8.3%) with *T. velifera* only. Occurrence of single-species and multiple-species infections is shown in Table 2. Confirmed multiple-species infections occurred in 39 (65%) of the specimens. Six specimens (10%) were positive for the *Theileria/Babesia* genus-specific probe only; no hybridisation occurred to species-specific probes on the RLB membrane (Table 2).

#### DISCUSSION

Four specimens (6.7%) were positive for *T. parva*. As ECF no longer occurs in South Africa, we assume that this result indicates the presence of buffalo-associated *T. parva*. Specimens were obtained from apparently healthy cattle only, which strongly suggests that these cattle are subclinical carriers of buffalo-associated *T. parva*.

Corridor disease has been regarded as self-limiting in cattle because they usually die before the parasite develops to the

Table 2: Occurrence of *Theileria* spp. and *Babesia* spp. infections in cattle along the northeastern edge of Hluhluwe-iMfolozi Park, KwaZulu-Natal, South Africa.

No. of species present	No. of specimens
1	15 (25.0 %)
2	9 (15.0 %)
3	26 (43.3 %)
4	4 (6.7 %)
<i>Theileria/Babesia</i> genus-specific probe positive only	6 (10.0 %)
Total	60 (100 %)

piroplasm stage, which is infective to ticks<sup>30</sup>. Experimental evidence suggests that some bovines survive the disease, however, and may serve as reservoirs of infection<sup>15</sup>. Furthermore, South Africa is considered free of *T. parva*, except in designated Corridor disease-infected areas including and bordering the Kruger National Park and Hluhluwe-iMfolozi Park. Therefore the national herd is essentially naïve and completely susceptible to *T. parva*<sup>39</sup>.

These results and those of a study on another cattle population<sup>39</sup>, suggest that cattle could be subclinical carriers of *T. parva* parasites in South Africa. A study should be carried out to determine whether these parasites could serve as a source of infection to disease-transmitting ticks. These findings would be very important, as they could indicate that current control regulations in South Africa may have to be revised. The assumption that only buffaloes are carriers of the parasite, and thus merely preventing contact with buffaloes may no longer be tenable. Therapeutic

treatment of Corridor disease is not allowed in South Africa as recovered intact cattle could develop a carrier status and could become infective to ticks<sup>35</sup>. If carriers are present and cattle-to-cattle transmission occurs, it could lead to the selection of a subpopulation of *T. parva* parasites that are better adapted to cattle as host. If this were to happen in South Africa, as is speculated is the case in East Africa<sup>2</sup>, it would impact negatively on the cattle industry.

The high prevalence of *T. mutans* infection (83.3 %) was not really surprising, as it has been shown that virtually all calves in endemic areas may be infected by the time they are 6 months old<sup>26</sup>. African buffalo also harbour *T. mutans*<sup>3,33</sup> and there is field and experimental evidence suggesting that strains of *T. mutans* from buffalo may be more pathogenic to cattle than those derived from cattle<sup>20</sup>. The prevalence of *T. velifera*, which is also known to infect buffalo<sup>37</sup>, was also quite high (70.0 %). This piroplasm is generally regarded as apathogenic in cattle<sup>21</sup>. *Theileria taurotragi*, which is mildly pathogenic in cattle but has been associated with cerebral theileriosis (so-called turning disease) was initially described from eland *Taurotragus oryx*<sup>9,18,19,22</sup>. The high prevalence of *Theileria* sp. (sable) (46.8 %) is interesting. This species, which has been reported from cattle in Tanzania, is incriminated in causing mortalities in sable antelope (*Hippotragus niger*) and possibly roan antelope (*Hippotragus equinus*)<sup>29</sup>, neither of which occurs in Hluhluwe-iMfolozi Park. It has also been reported from African buffalo, blue wildebeest (*Connochaetes taurinus*), klipspringer (*Oreotragus oreotragus*) and reedbuck (*Redunca arundinum*)<sup>29</sup>, all of which occur in Hluhluwe-iMfolozi Park. A closely related *Theileria* species has been reported from dogs in KwaZulu-Natal<sup>25</sup>.

Two of the specimens were positive for *B. bovis*, indicating a degree of the additional challenge the herd may be exposed to. A third sample was positive for *B. bigemina*. This extremely low prevalence of *Babesia* spp. indicates effective tick control in the area.

One of the specimens was positive for *B. rossi*. This parasite had previously only been found in canids<sup>23,24,31</sup> and thus this result may have been due to contamination. With PCR product contamination, however, more than 1 false positive result would have been expected. Accidental transmission of parasites to aberrant hosts is possible. These parasites may survive and be detected, although it is unlikely that the parasite would be able to reproduce and cause disease in the accidental host. It is possible that accidental

transmission could have produced a positive result. *Theileria equi*, which had not been described previously as a parasite of canids, has been found in asymptomatic dogs<sup>6</sup>. It was concluded that infection of dogs with *T. equi* is not unlikely to happen and that molecular methods can lead to a correct identification of piroplasmids in accidental hosts. Similarly, in this study, accidental transmission of *B. rossi* in cattle probably occurred.

A fairly large percentage (10 %) of the specimens were positive for the *Theileria/Babesia* genus-specific probe only, but no hybridisation occurred to species-specific probes on the RLB membrane. This could indicate that there may be novel *Theileria* or *Babesia* species in cattle or variants of species present in these specimens. Alternatively, it could indicate the presence of a known species for which no probe was included on the blot. Not all probes that are specific for all the *Theileria* and *Babesia* species that could occur in cattle were used in this study; for example, *B. occultans* was not included. Alternatively, the concentration of species-specific DNA may have been too low to be detected in the hybridisation reaction. Sequence analysis could be carried out on these specimens that hybridised only to the *Theileria* and *Babesia* genus-specific probe to determine the nature of these specimens.

Two of the 60 specimens were positive for *Trypanosoma theileri* and 1 was positive for *T. vivax*. Results of a previous survey of the same area showed that bovine trypanosomosis is prevalent in the vicinity of the Hluhluwe-iMfolozi Park<sup>41</sup>. According to that survey, in which anaemic animals had been selected, 60.5 % of the specimens were positive for *Trypanosoma congolense*<sup>41</sup>. *Trypanosoma vivax* is one of the species responsible for causing nagana, while *Trypanosoma theileri* (subgenus *Megatrypanum*) is generally regarded as non-pathogenic.

In summary, we determined occurrence of haemoprotozoa in 60 cattle at the wildlife/livestock interface in northern KwaZulu-Natal. The most important finding was that 4 cattle (6.7 %) were infected with *T. parva*. The presence of subclinical *T. parva* carriers, which were also recently found in another cattle population<sup>39</sup> in KwaZulu-Natal, would suggest that the premises on which current Corridor-disease-control strategies in South Africa are based may have to be reassessed.

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