Tick-borne blood parasites in nyala (*Tragelaphus angasii*, Gray 1849) from KwaZulu-Natal, South Africa

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ABSTRACT

A total of 97 blood samples of nyala (*Tragelaphus angasii*, Gray 1849) from South Africa were tested for the presence of tick-borne haemoparasites by means of polymerase chain reaction (PCR) and reverse line blot (RLB) hybridisation. The majority of blood samples contained several different haemoparasites, often in combination. Prevalent haemoparasites were *Theileria* sp. (kudu), *T. buffeli*, *T.* sp. (sable), *T. bicornis*, *Ehrlichia* sp. Omatjenne, *Anaplasma marginale* and *A. bovis*. This serves as the first report of *T.* sp. (kudu), *T. buffeli*, *T. bicornis*, *Ehrlichia* sp.

Omatjenne, *A. marginale* and *A. bovis* in nyala, who seem to carry multiple haemoparasites without ill effect.

1. INTRODUCTION

Many haemoparasites have been identified in domestic and wild animals since the late 1800s. Piroplasms (*Babesia* and *Theileria* species), *Anaplasma* species and *Ehrlichia ruminantium* contribute to huge economic losses in the African livestock industry (Uilenberg 1995). Although tick-borne haemoparasites have also been implicated in losses amongst wild animals, including endangered species (Kuttler 1984; Peter et al. 2002; Penzhorn 2006), the epidemiology and phylogeny of piroplasms of wildlife are still largely unknown. New techniques such as polymerase chain reaction (PCR) and reverse line blot (RLB) hybridisation (Gubbels et al. 1999; Bekker et al. 2002) make surveying and typing of piroplasms and other haemoparasites easier and more reliable than the traditionally used blood-smear methods. In this study, these new techniques were utilised to survey the occurrence of piroplasms, *Ehrlichia* and *Anaplasma* species in nyala (*Tragelaphus angasii*, Angas 1849), a medium-sized antelope with a fairly restricted distribution in south-eastern Africa (north-eastern South Africa, south-eastern Zimbabwe, south-central Mozambique and southern Malawi) (Skinner and Chimimba 2005).

In the only published paper referring to nyala blood parasites, "*Theileria*-like piroplasms" were reported from 4/16 blood smears (Keep, 1971). Piroplasms have been reported from greater kudu (*Tragelaphus strepsiceros*) (Neitz, 1931, 1933, 1957; Nijhof et al. 2005), bushbuck (*Tragelaphus scriptus*) (Ross 1911, in Wenyon 1926;

Neitz, 1931, 1933) and grey duiker (*Sylvicapra grimmia*) (Bettencourt and Borges 1909, in Wenyon 1926; Neitz and Thomas, 1948; Nijhof et al. 2005), which occur in the same bushveld and riverine habitat as nyala.

Nyala are popular game-ranch animals (Pfitzer and Kohrs, 2005). Large numbers are captured annually and handled individually for translocation, which facilitates surveying for blood parasites. Nyala naturally carry large tick burdens (Horak et al. 1983; Horak et al. 1995) and many haemoparasites are tick-transmitted, therefore it was likely that nyala were infected. High losses of nyala after translocation are not uncommon and haemoparasites, together with translocation stress, could potentially be the cause for these losses as was possibly the case in greater kudu (Nijhof et al. 2005) and black rhinoceros (*Diceros bicornis*) (Nijhof et al. 2003).

2. MATERIAL AND METHODS

2.1 Blood samples

Blood samples (n = 97) were obtained from 90 fully grown and 7 juvenile nyala during routine capture procedures on four game ranches in the Pongola area, northern KwaZulu-Natal, in 2007 and 2008. Samples were collected on Whatman® filter paper grade F 572-02 (Merck). The blood spots were stored in a dry, dark place for several months until they could be transported to the Molecular Biology Laboratory, Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria.

2.2 DNA Extraction

DNA was extracted from dried blood spots using the QIAamp® DNA Mini kit (QIAGEN, Southern Cross Biotechnologies) following the manufacturers' instructions. Extracted DNA was eluted in 100 µl elution buffer and stored at 4°C until further analysis.

2.3 Polymerase Chain Reaction

PCR was conducted as described by Nijhof et al. (2003) and Nijhof et al. (2005). Briefly, the V4 hypervariable area of the 18S ribosomal RNA (rRNA) gene was amplified using the *Theileria* and *Babesia* genus-specific primers RLB F2 (5'-GAC ACA GGG AGG TAG TGA CAA G-3') and biotin-labelled RLB R2 (5'-Biotin-CTA AGA ATT TCA CCT CTA ACA GT-3'). For *Ehrlichia* and *Anaplasma* species, a 492 to 498 bp fragment of the hypervariable V1 region of the 16S rRNA gene was amplified by PCR using Ehr-F (5'-GGA ATT CAG AGT TGG ATC MTG GYT CAG) as forward primer as described by Schouls et al. (1999) and the biotin-labelled Ehr-R reverse primer (5'-Biotin-CGG GAT CCC GAG TTT GCC GGG ACT TYT TCT) as described by Bekker et al. (2002). These primers have the same melting temperatures and therefore the thermocycler program for *Babesia* / *Theileria* and *Ehrlichia* / *Anaplasma* is the same.

The PCR reaction mixture consisted of 12.5 µl of Platinum® Quantitative PCR SuperMix-UDG (Invitrogen, The Scientific Group, South Africa), 20 pmol (0.25 µl) of each primer, 5 µl of DNA to a total volume of 25 µl. Positive and negative controls were included in each batch of samples. The positive control consisted of DNA extracted from a blood sample confirmed positive for several *Theileria, Babesia*, *Ehrlichia* and *Anaplasma* species. The negative control consisted of molecular grade

water. The Gene Amp®PCR System 9700 (Applied Biosystems, South Africa) and the 2720 Thermal Cycler (Applied Biosystems, South Africa) were used to amplify the DNA. A touchdown PCR thermocycler program was followed.

2.4 Reverse Line Blot hybridization assay

The PCR products were analysed using the RLB hybridisation technique (Gubbels et al. 1999). Controls for *Ehrlichia / Anaplasma* species and for *Theileria / Babesia* species used in the RLB were plasmid controls and supplied with the kit by Isogen Life Science (the Netherlands). Two different membranes were used: the commercially available TBD-RLB membrane, supplied by Isogen Life Science (the Netherlands), and an in-house prepared membrane. Genus- and species-specific probes present on the in-house membrane are listed in Table 1. For preparation of the in-house membrane, the species-specific oligonucleotides were diluted in 150 µl 0.5 M $NaHCO_3$. The membrane was then marked and incubated for 10 min in 16% EDAC at room temperature. It was then rinsed with demineralised water.

3. RESULTS

Ninety-three of the 97 blood specimens (95.9%) gave positive reactions for haemoparasites on RLB. Most positive samples were multiple infections. Adults as well as juveniles tested positive. The piroplasms identified (in decreasing order of prevalence) were (Fig. 1): *Theileria* sp. (kudu) (87/97; 89.7%), *T. buffeli* (85/97; 87.6%), *Theileria* sp. (sable) (57/97; 58.8%) and *T. bicornis* (51/97; 52.6%). No *Babesia* spp were recorded. Four specimens (4.1%) were positive for the *Theileria / Babesia* genus-specific probe only, but for none of the species-specific probes.

Fewer animals were infected with rickettsias (Fig. 2): 32/97 (33.0%) were positive for *Ehrlichia* sp. Omatjenne, an apathogenic species. None was positive for *E. ruminantium*, the causative agent of heartwater. Thirteen animals (13.4%) were positive for *A. marginale*. and 11/97 (11.3%) were positive for *A. bovis*. Seventeen (17.5%) of the specimens showed a signal at the *Ehrlichia / Anaplasma* genus specific probe only, but none at any of the species-specific probes.

When parasite species could be determined, the combinations were as follows: only one animal carried only one haemoparasite species – *Ehrlichia* sp. Omatjenne. Twelve animals were carriers of two haemoparasite species, in most cases this was a combination of *Theileria* sp. (kudu) and *T. buffeli*. Seventeen animals tested positive for three different haemoparasite species but most animals (n = 36) tested positive for a combination of 4 haemoparasite species. Mostly, these were combinations of *T. buffeli*, *Theileria* sp. (kudu), *T. bicornis* and *Theileria* sp. (sable). Fifteen animals tested positive for five different haemoparasite species and six animals tested for six different haemoparasite species. The most common combination of six haemoparasites was *T. buffeli*, *Theileria* sp. (kudu), *T. bicornis*, *Theileria* sp. (sable), *Ehrlichia* sp. Omatjenne and *Anaplasma marginale*.

4. DISCUSSION

As sampled animals appeared healthy and translocated well, nyala seem to carry multiple haemoparasites without ill effect. Most haemoparasites carried by nyala are not known to be pathogenic. The high prevalence of haemoparasites in nyala was not unexpected, in view of the high tick burdens that these antelope are exposed to (Baker & Keep 1970; Horak et al. 1983; Horak et al. 1995).

4.1 Theileria sp. (kudu)

All but 10 of the specimens were positive for *Theileria* sp. (kudu), which has been incriminated in causing mortality in greater kudu shortly after translocation. *Theileria* sp. (kudu) is not known to be pathogenic to domestic animals and other wildlife species and phylogenetically is closest related to a *Theileria* species that was isolated from *Bos indicus* cattle in Thailand (Nijhof et al. 2005). It is not known whether *Cytauxzoon strepsicerosi*, described from greater kudu (Neitz 1957), is the same taxon as *Theileria* sp. (kudu), which is reported from nyala for the first time. Nyala are known to suffer from translocation stress and high, often unexplained, losses can occur after translocation. If captive or recently translocated nyala show typical signs of theileriosis (i.e., anaemia, lymph node enlargement, petechiae, splenomegaly or lung oedema), clinical theileriosis due to *Theileria* sp. (kudu) should be considered as a differential diagnosis as greater kudu and nyala are both *Tragelaphus* species. Therefore it can be assumed that the parasite might have the same effect on both species. The vector of *Theileria* sp. (kudu) is yet unknown.

4.2 Theileria buffeli

Theileria buffeli occurred in 87.6% of the specimens. The *T. buffeli* group consists of mainly benign bovine parasites that are common in cattle worldwide, with prevalences ranging from 30% to nearly 100% (Stewart et al. 1992; Georges et al. 2001; Cossio-Bayugar et al. 2002; Garcia-Sanmartin et al. 2006; Salih et al. 2007; Altay et al. 2008). It was found in 23 of 24 African buffalo (*Syncerus caffer*) tested in the Kruger National Park, South Africa (Allsopp et al. 1999). The world-wide occurrence of parasites of the *T. buffeli* group can be explained by the broad range of

tick vectors. *Haemophysalis*, *Dermacentor* as well as *Amblyomma* species have been implicated in the transmission of these parasites (Gubbels et al. 2000). The *T. buffeli* group is very diverse but consists mainly of buffalo-derived parasites that have adapted to cattle. *Theileria sergenti,* a member of the *T. buffeli* group is a pathogenic parasite that occurs in sheep in eastern Asia (Lawrence 2005). This is the first report of *T. buffeli* from an antelope species. Due to its wide distribution however, it is likely that *T. buffeli* may be identified from other African antelope species.

4.3 Theileria sp. (sable)

Theileria sp. (sable), found in 58.8% of specimens, causes fatal clinical disease in roan antelope (*Hippotragus equinus*) and sable antelope (*Hippotragus niger*) in South Africa with clinical signs including anaemia and icterus (Nijhof et al. 2005). *Theileria* sp. (sable) has also been isolated from healthy animals, such as African short-horn cattle, African buffalo, blesbok (*Damaliscus pygargus*), blue wildebeest (*Connochaetus taurinus*), klipspringer (*Oreotragus oreotragus*) and common reedbuck (*Redunca arundinum*) (Nijhof et al. 2005). A closely related species was isolated from red hartebeest (*Alcelaphus buselaphus caama*) in Namibia (Spitalska et al. 2005). *Theileria* sp. (sable) is closest related to *T. separata*, which infects domestic small ruminants. The main vectors are possibly *Rhipicephalus evertsi evertsi* and *R. appendiculatus* (Nijhof et al. 2005).

4.4 Theileria bicornis

Theileria bicornis, carried by 52.6% nyalas in this survey, was originally described from healthy black rhinoceroses in South Africa and is not known to be pathogenic (Nijhof et al. 2003). *Theileria bicornis* was also described in 36.4% of white

rhinoceroses (*Ceratotherium simum*) from the Kruger National Park and in 3.8% of cattle tested in Uganda (Govender 2009, Muhanguzi et al. 2010). This is the first report of *T. bicornis* in nyala and although not much is known about this piroplasm, this finding shows that *T. bicornis* has a broad host range and crosses the species barrier. *Theileria bicornis* is closely related to *T. youngi* and *T. equi* (Nijhof et a. 2003). The tick vectors are unknown at this stage.

4.5 Ehrlichia. sp. Omatjenne

Although none of the specimens was positive for *E. ruminantium*, 33.0% carried *Ehrlichia sp.* Omatjenne, mostly as mixed infections with other haemoparasites. This *Ehrlichia*-like agent, initially isolated from *Hyalomma truncatum* (Du Plessis 1990), is generally thought to be apathogenic. After several passages of this agent through *Amblyomma* ticks, however, sheep developed severe signs of disease similar to heartwater (Du Plessis 1990). This is the first time *E.* sp. Omatjenne has been found in nyala.

4.6 Anaplasma marginale

Anaplasma marginale, the causative agent of gallsickness / bovine anaplasmosis, occurred in 13.4% of specimens. This disease occurs endemically in most cattle-farming areas in southern Africa (Potgieter & Stoltsz 2005). Most of the farms on which nyala were captured in the Pongola area had been used for cattle ranching not more than 5–7 years previously or share a boundary with cattle-grazing areas. *Anaplasma marginale* has been reported from various wildlife species but this is the first report of *A. marginale* in nyala. The parasite was implicated in the death of a giraffe (*Giraffa camelopardalis*) (Augustyn & Bigalke 1974), but generally does not

seem to cause clinical signs in wildlife. A grey duiker infected with *A. marginale* developed an inapparent infection (Neitz & Du Toit 1932). On blood smear examination, sable antelope, blesbok, blue wildebeest and black wildebeest (*Connochaetes gnou*) were found to be carriers of *A. marginale* (Thomas et al. 1982; Kuttler 1984). High seroprevalence of *Anaplasma* species, ranging from 75 to 100%, was found in wildlife at the livestock–wildlife interface in Kenya (Ngeranwa et al. 2008). Species examined were eland, blue wildebeest, kongoni (*Damaliscus korrigum*), impala (*Aepyceros melampus*), Thomson's gazelle (*Gazella thomsonii*), Grant's gazelle (*Gazella granti*), giraffe and plains zebra (*Equus quagga*). This indicates that wildlife may play a significant role in the epidemiology of *Anaplasma* organisms and that wildlife could serve as a reservoir for infection of cattle.

4.7 Anaplasma bovis

Anaplasma bovis, previously described as *Ehrlichia bovis* but reclassified by Dumler et al. (2001), was found in 11.3% of specimens. *Anaplasma bovis*, the cause of bovine ehrlichiosis (called Nofel in West Africa), has also been reported from South America, West, Central and southern Africa and India (Sumption & Scott 2005). *Anaplasma bovis* was also recently isolated from cottontail rabbits (*Sylvilagus floridanus*) in North America (Goethert & Telford 2003) and from wild deer in Japan (Kawahara et al. 2006). Serological cross-reaction with *E. ruminatium* has been reported (Dumler et al. 2001). *Anaplasma bovis* is not uncommon in South Africa and the known vector is *Rhipicephalus appendiculatus,* but it is possibly also transmitted by *Amblyomma*, *Hyalomma* and other *Rhipicephalus* species (Sumption & Scott 2005). Tonetti et al. (2009) detected *A. bovis* in a *Rhipicephalus evertsi evertsi* tick from a gemsbok (*Oryx gazella gazella*) that was collected in the Freestate Province of

South Africa. This is the first report of *A. bovis* in nyala and as pointed out previously, wild ruminants could very well play a reservoir role in the transmission cycle of *Anaplasma* species, therefore increasing difficulties to control the relevant diseases in cattle (Tonetti et al. 2009).

Animals showed a signal at the *Ehrlichia / Anaplasma* genus-specific probe in 17 cases, without showing a signal at any of the species-specific probes. Weak signals could indicate that there were not enough amplicons in these samples to give a species-specific signal. It could also mean that new species or variant of species of *Anaplasma* or *Ehrlichia* could be present in these samples, which would need further investigation.

From this study one can conclude, that nyala – although not clinically affected – commonly carry multiple infections of various *Theileria* species, as well as of *Anaplasma* species and *Ehrlichia* species. Haemoparasites identified from nyala in this study were *Theileria* sp. (kudu), *T. buffeli*, *Theileria* sp. (sable), *T. bicornis*, *Ehrlichia* sp. Omatjenne, *A. marginale* and *A. bovis*. Most of the organisms were reported in nyala for the first time. The fact that some of the genus-specific probes showed up positive without any of the species-specific probes showing a match, could very well mean that as yet unknown haemoparasites may have been present in these samples. This is supported by the number of new haemoparasites that have recently been identified (Nijhof et al. 2003; Nijhof et al. 2005; Oosthuizen et al. 2008; Oosthuizen et al. 2009).

Whether parasitaemias are high enough for engorging ticks to become infected, thereby contributing to spreading these haemoparasites, would need further investigation.

Juvenile animals already carried multiple infections with haemoparasites. This is either an indication that nyala are exposed to these organisms at a young age or it could indicate that they have been infected in uteri or via milk.

Following these results, it should be stressed that when translocating nyala or any wild animal, measures should be taken to avoid introducing piroplasms and their tick vectors into naive populations or to areas where they did not occur previously. This is especially important taking into consideration that nyala carry *Theileria* sp. (sable). Nyala are often delivered into camp systems and bred under intensive conditions together with other valuable species, such as sable antelope or roan that are potentially susceptible to disease caused by *Theileria* sp. (sable) (Nijhof et al. 2005).

Not only can piroplasms develop into a problem, if they are introduced into a naive population, they can also become cause of disease in animals that are usually asymptomatic carriers if these animals are stressed. The subclinical infection with *Theileria* sp. (kudu) could be contributing to the sporadic high losses of nyala shortly after translocation, similar to mortalities in greater kudu due to theileriosis after translocation (Nijhof et al. 2005).

This study contributed to the expansion of the known host ranges of several haemoparasites, which shows that most piroplasms are not strictly species-specific

and that multiple infections are not uncommon. Altogether, the role that piroplasms play is by no means clear and apart from the fact that some species are obviously pathogenic, non-pathogenic piroplasms could also play a positive role – for example, they might aid in the protection of an animal from infection with pathogenic piroplasms.

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Table 1: Genus- and species-specific probes present on the in-house prepared membrane. Symbols indicating degenerate positions: R = A/G, W = A/T, K = G/T

Species	Probe Sequence from 5' to 3'
Ehrlichia / Anaplasma genus-specific probe	GGG GGA AAG ATT TAT CGC TA
Anaplasma centrale	TCG AAC GGA CCA TAC GC
Anaplasma marginale	GAC CGT ATA CGC AGC TTG
Anaplasma phagocytophilum	TTG CTA TAA AGA ATA ATT AGT GG
Ehrlichia ruminantium	AGT ATC TGT TAG TGG CAG
Anaplasma bovis	GTA GCT TGC TAT GRG AAC A
Ehrlichia chaffeensis	ACC TTT TGG TTA TAA ATA ATT GTT
Ehrlichia sp. Omatjenne	CGG ATT TTT ATC ATA GCT TGC
Ehrlichia canis	TCT GGC TAT AGG AAA TTG TTA
Theileria / Babesia genus-specific probe	TAA TGG TTA ATA GGA RCR GTT G
Theileria genus-specific probe	ATT AGA GTG CTC AAA GCA GGC
Babesia genus-specific probe 1	ATT AGA GTG TTT CAA ACA GGC
Babesia genus-specific probe 2	ACT AGA GTG TTT CAA ACA GGC
Babesia felis	TTA TGC GTT TTC CGA CTG GC
Babesia divergens	ACT RAT GTC GAG ATT GCA C
Babesia microti	GRC TTG GCA TCW TCT GGA
Babesia bigemina	CGT TTT TTC CCT TTT GTT GG
Babesia bovis	CAG GTT TCG CCT GTA TAA TTG AG
Babesia rossi	CGG TTT GTT GCC TTT GTG
Babesia canis canis	TGC GTT GAC GGT TTG AC
Babesia canis vogeli	AGC GTG TTC GAG TTT GCC
Babesia major	TCC GAC TTT GGT TGG TGT
Babesia bicornis	TTG GTA AAT CGC CTT GGT C
Babesia caballi	GTT GCG TTK TTC TTG CTT TT
Theileria sp. (kudu)	CTG CAT TGT TTC TTT CCT TTG
Theileria sp. (sable)	GCT GCA TTG CCT TTT CTC C
Theileria bicornis	GCG TTG TGG CTT TTT TCT G
Theileria annulata	CCT CTG GGG TCT GTG CA
Theileria buffeli	GGC TTA TTT CGG WTT GAT TTT
Theileria sp. (buffalo)	CAG ACG GAG TTT ACT TTG T
Theileria mutans	CTT GCG TCT CCG AAT GTT
Theileria parva	GGA CGG AGT TCG CTT TG
Theileria taurotragi	TCT TGG CAC GTG GCT TTT
Theileria velifera	CCT ATT CTC CTT TAC GAG T
Theileria equi	TTC GTT GAC TGC GYT TGG
Theileria lestoquardi	CTT GTG TCC CTC CGG G
Theileria separata	GGT CGT GGT TTT CCT CGT
Theileria ovis	TTG CTT TTG CTC CTT TAC GAG
Babesia sp. (sable)	GCT GCA TTG CCT TTT CTC C
Babesia gibsoni	CAT CCC TCT GGT TAA TTT G

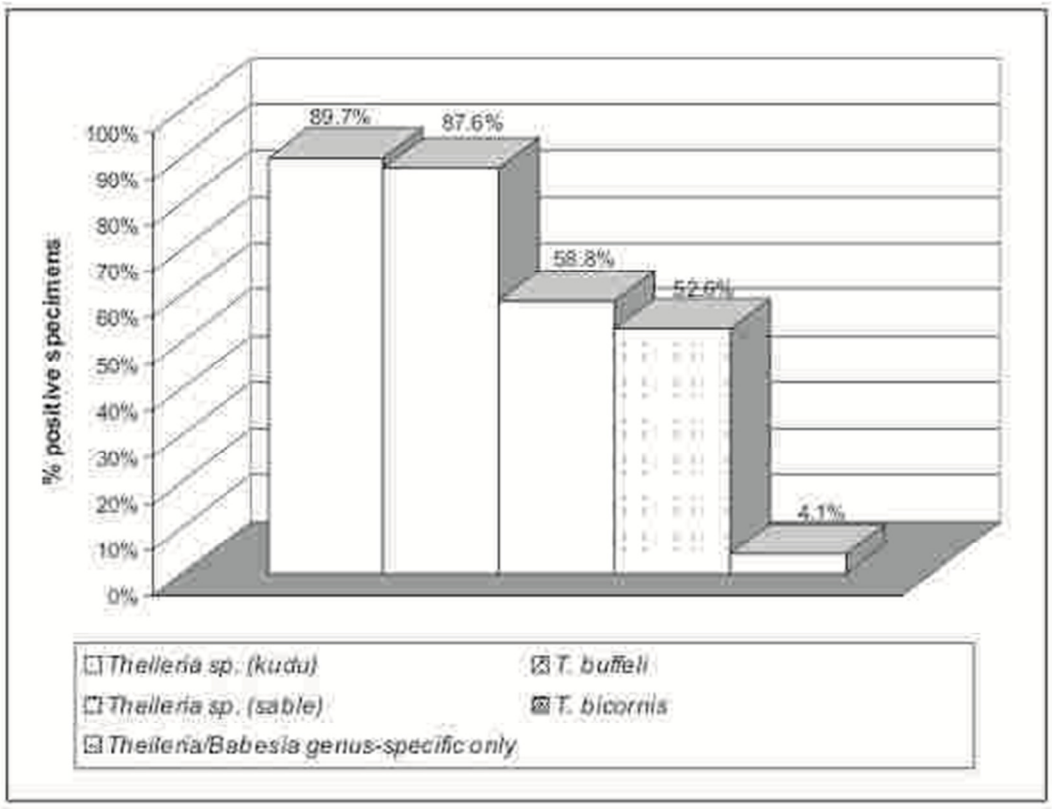

Figure 1: Occurrence of *Theileria* and *Babesia* species infections in nyala specimens as determined by the Reverse Line Blot hybridization assay.

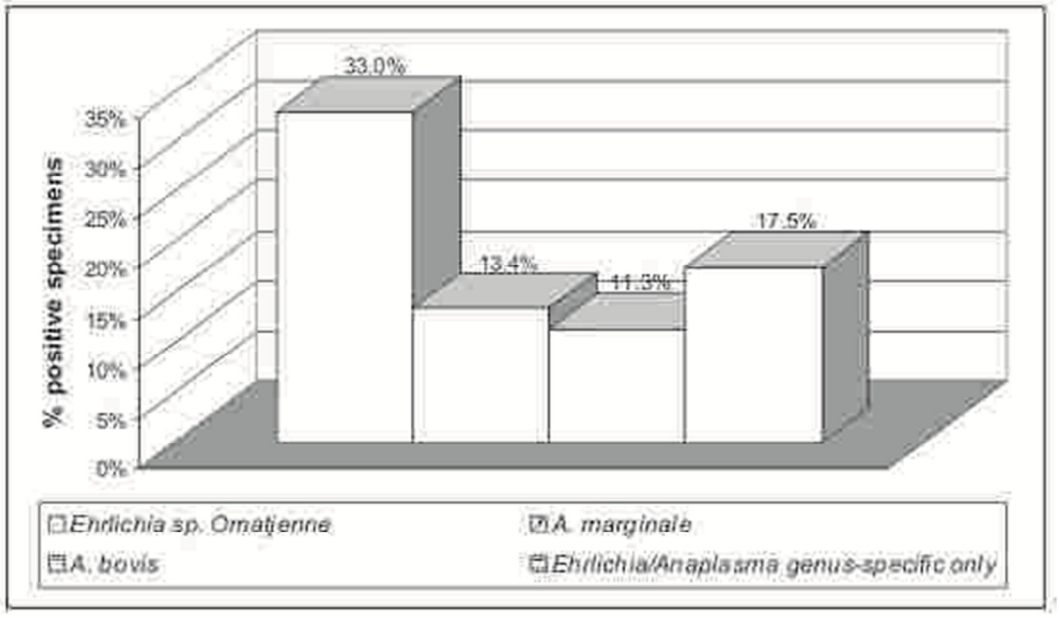

Figure 2: Occurrence of *Ehrlichia* and *Anaplasma* species infections in nyala specimens as determined by the Reverse Line Blot hybridization assay.