

**Occurrence of tick-borne haemoparasites
in nyala (*Tragelaphus angasi*) in
KwaZulu-Natal and Eastern Cape Province,
South Africa**

by
Silke Pfitzer

Submitted in partial fulfilment of the requirements for the
degree

Master of Science (Veterinary Tropical Diseases)

in the Faculty of Veterinary Science,
University of Pretoria

June 2009



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ACKNOWLEDGEMENTS

I would like to thank the staff of the Department of Veterinary Tropical Diseases of the University of Pretoria, especially my supervisor, Prof. Banie Penzhorn, and my co-supervisor Dr. Marinda Oosthuizen, for their input and suggestions.

I would like to also give a special thank-you to Anne-Mari Bosman, who taught me the laboratory methods necessary for my project and always assisted with technical support. I would also like to thank Milana Troskie who kindly sacrificed her RLB membrane for my project and also assisted with technical support. Both are from the Department of Veterinary Tropical Diseases of the University of Pretoria.

I would additionally like to thank the librarians and their staff – Mrs. Antoinette Lourens from the Faculty of Veterinary Science, University of Pretoria, and Mr. David Swanepoel from the library of the Onderstepoort Veterinary Institute.

A big thank-you also to Dr. Luis Amaral for providing samples from the Eastern Cape Province and to Elise Klopper, who accommodated me so kindly during the times that I had to stay in Pretoria.

A special thanks to my husband, Ian Colenbrander, and the staff of Chui Wildlife Services for assisting with lots of samples from the Pongola area and for providing me with time and encouragement to carry out this study.

This thesis was supported by a postgraduate bursary from the University of Pretoria and by the National Research Foundation Grant (GUN 44403) to B.L. Penzhorn.

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Co-supervisor: Dr M C Oosthuizen
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Abstract

A total of 143 blood samples of nyala (*Tragelaphus angasi*) from two regions in South Africa were tested for the presence of tick-borne haemoparasites by means of polymerase chain reaction (PCR) and reverse line blot (RLB) hybridisation. While most blood samples taken in EDTA blood turned out negative for the presence of haemoparasites, the majority of blood samples collected on Whatman® filter paper contained several different haemoparasites, often in combination. Samples from the Eastern Cape Province as well as from KwaZulu-Natal turned out positive. Prevalent haemoparasites were *Theileria* sp. (kudu), *T. buffeli*, *T. bicornis*, *Theileria* sp. (sable), *T. taurotragi*, *Ehrlichia* sp. Omatjenne, *Anaplasma bovis* and *A. marginale*. This serves as the first report of *T. buffeli*, *T. sp.* (kudu), *T. bicornis*, *T. taurotragi*, *Ehrlichia* sp. Omatjenne, *A. marginale* and *A. bovis* in nyala.

1. INTRODUCTION

During the past eighty years, many haemoparasites have been identified in domestic as well as in wild animals. Piroplasms (*Babesia* and *Theileria* species), *Anaplasma* species, as well as *Ehrlichia ruminantium*, the causative organism of heartwater, contribute to huge economic losses in the African livestock industry (Uilenberg 1995). Tick-borne haemoparasites have also been implicated in losses amongst wild animals, some of which were endangered species (Kuttler 1984; Peter, Burridge & Mahan 2002; Penzhorn 2005; Penzhorn 2006). Despite these facts, not much is known about the epidemiology and phylogeny of piroplasms. New techniques such as polymerase chain reaction (PCR) and reverse line blot (RLB) hybridisation have been developed during the past ten years (Gubbels, De Vos, Van der Weide, Viseras, Schouls, De Vries & Jongejan 1999; Bekker, De Vos, Taoufik, Sparagano & Jongejan 2002). These will make surveys and typing of piroplasms and other haemoparasites easier and more reliable than the traditionally used blood smear methods. This thesis utilised these new methods to carry out a survey on the occurrence of piroplasms, *Ehrlichia* and *Anaplasma* species in nyala (*Tragelaphus angasi*). This survey was part of research carried out by the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria, in an attempt to shed more light on the epidemiology of piroplasms and on the role played by various wild animal species in their epidemiology (Penzhorn 2006).

2. Literature Review

Many different tick-borne haemoparasites have thus far been discovered in domestic species as well as in wildlife worldwide. The effects of haemoparasites often depend on the host species and immunity of the host and can vary from development of severe disease due to the infection with haemoparasites to a completely inapparent infection without any signs of disease.

2.1. PIROPLASMS: *BABESIA* AND *THEILERIA* SPECIES

Piroplasms are defined as non-pigment-forming haemoparasites (Uilenberg 2006). To date, two genera of piroplasms have been defined, namely *Babesia* and *Theileria*. *Babesia* are defined as parasites that enter directly into red blood cells of the host after injection. In contrast, *Theileria* sporozoites do not initially infect red blood cells but penetrate a lymphocyte or macrophage in which they develop into schizonts. The merozoites released from the schizonts then enter red blood cells where they grow into piroplasms and multiply by budding into four daughter cells, thus generating the form of a maltese cross. The development inside the tick vector also differs between *Babesia* and *Theileria*. Ticks become infected with piroplasms when ingesting infected red blood cells. Piroplasms in the tick develop into male and female gametes. Microgametes and macrogametes fuse to form zygotes, which are motile. The zygotes of *Babesia* multiply and vermicles invade numerous organs of the tick, including the ovaries. Here the infection passes into the egg and to the next tick generation. This is called transovarial transmission. Certain species of *Babesia* can persist over several tick generations, even without new infection (Friedhoff 1988). The zygotes of *Theileria*, in contrast, do not multiply but invade the haemolymph of the tick where they go towards the salivary glands. When the next instar of the vector attaches to a new host, sporogony and maturation of the sporozoites in the salivary glands occur and transmission takes place by injection of infected saliva. This is called transstadial transmission. The tick loses its theilerial infection after having transmitted it and the infection does not persist to the next instar or the next generation. For example, when the larva becomes infected, the nymph will be infective and when the nymph becomes infected, the adult will be infective (Uilenberg 2006). Older literature often mentions a third category of piroplasms, *Cytauxzoon*. For the time being, Cytauxzoonosis is classified as Theileriosis, as *Cytauxzoon* and *Theileria* might be synonymous – according to Levine (1971, cited by Carmichael & Hobday 1975).

Not every piroplasm can be easily grouped as some seem to have attributes of both – *Babesia* as well as *Theileria* (Allsopp, Cavalier-Smith, De Waal & Allsopp 1994; Uilenberg 2006). Today, definition of species of piroplasms relies heavily on molecular characterisation and the validity of several species named in the past is being questioned (Penzhorn 2006).

Piroplasms have been isolated from many different vertebrates. While some of the piroplasms known in domestic and wild mammals seem species specific, it was proven that others are able to cross the species barrier. These include *Theileria parva*, *Theileria taurotragi* and *Babesia bigemina* as well as *Theileria equi* (Grootenhuis, Morrison, Karlstad, Sayer, Young, Murray & Haller 1980; De Waal & Van Heerden 1994; De Vos, de Waal & Jackson. 2005; Lawrence & Williamson 2005; Lawrence, Perry & Williamson 2005b). *Babesia divergens* has even been classified as a zoonosis (Zintl, Mulcahy, Skerrett, Taylor & Gray 2003).

In domestic mammals, piroplasms cause some of the most economically important diseases such as babesiosis and theileriosis in cattle (BurrIDGE 1975; De Vos *et al.* 2005; Lawrence & Williamson 2005; Lawrence, Perry & Williamson 2005a; Lawrence *et al.* 2005b) and babesiosis in horses (De Waal & Van Heerden 1994).

African buffalo (*Syncerus caffer*) are known to be asymptomatic hosts of *T. parva*, which is transmitted mainly by the brown ear tick (*Rhipicephalus appendiculatus*). In domestic cattle, *T. parva* causes diseases such as East Coast fever, Corridor disease and Zimbabwe theileriosis (Lawrence *et al.* 2005a; Lawrence *et al.* 2005b; Lawrence, Perry & Williamson 2005c). African buffalo can be artificially infected with *B. bigemina* – another pathogenic haemoparasite of cattle (De Vos *et al.* 2005). Waterbuck (*Kobus defassa*) were also found to be asymptomatic carriers of *T. parva* (Stagg, Bishop, Shaw, Wesonga, Orinda, Grootenhuis, Molyneux & Young 1994).

Eland (*Taurotragus oryx*) have been identified as carriers of *T. taurotragi*, which can lead to clinical disease in eland as well as in cattle where it causes turning sickness (Grootenhuis *et al.* 1980; Lawrence & Williamson 2005). *Theileria taurotragi* is transmitted by *Rhipicephalus appendiculatus*, *R. evertsi evertsi*, *R. pulchellus* and *R. zambeziensis* (Lawrence, De Vos & Irvin 1994).

Neitz (1931) reported piroplasms in blood smears of 16 of 55 plains (or Burchell's) zebra (*Equus burchelli*) examined in Zululand. Plains zebra and Cape mountain zebra (*Equus zebra zebra*) are asymptomatic carriers of *B. equi* (today also called *T. equi*), which causes babesiosis in domestic horses (Young, Zumpt, Boomker, Penzhorn & Erasmus 1973; De Waal & Van Heerden 1994; Zweygarth, Lopez-Rebollar & Meyer 2002).

Sable antelope (*Hippotragus niger*) have reportedly died of clinical babesiosis following infection with *B. irvinesmithi* (Martignalia 1930; Thomas, Wilson & Mason 1982; McInnes, Stewart, Penzhorn & Meltzer 1991; Hove, Sithole, Munodzana & Masaka 1998). The first case was reported by Martignalia (1930) who carried out a post mortem on a sable antelope that had been translocated to Johannesburg Zoo six weeks previously. Babesias were found

in the erythrocytes of smears taken from spleen and liver stained with Giemsa. In a survey carried out in South Africa, seven of 124 blood smears of sable antelope were found to contain *Babesia* spp., possibly *B. irvinesmithi* (Thomas, Wilson & Mason 1982). Five of these smears had been taken from sable antelope carcasses found in the veld. Seventy of the 124 smears contained piroplasms resembling *Theileria* spp. Attempts to infect intact and splenectomised sable as well as cattle by sub-inoculation of blood from infected animals were not successful. The experimental infection of two splenectomized sable with *B. bovis* and *B. bigemina* also failed. It was therefore concluded that *B. irvinesmithi* possibly is a *Babesia* species of the sable antelope. Recently a further *Babesia* species – *Babesia* sp. (sable) – was described from a sable antelope that died during immobilization after showing signs of disease (Oosthuizen, Zweygarth, Collins, Troskie & Penzhorn 2008). A *Theileria* species characterized as *Theileria* sp. (sable) was isolated from a blood sample of a clinically ill sable antelope (Nijhof, Pillay, Steyl, Prozesky, Stoltsz, Lawrence, Penzhorn & Jongejan 2005)

Roan antelope (*Hippotragus equinus*) seem to be very susceptible to a *Theileria* species that was named *Theileria hippotragi* (Steyl, Lawrence, Prozesky, Stoltsz & Penzhorn 2004). *Theileria* isolated from roan antelope by Nijhof *et al.* (2005) were characterized as *Theileria* sp. (sable). Theileriosis in roan antelope has major implications for many roan antelope captive-breeding projects (Steyl *et al.* 2004). Nijhof *et al.* (2005) also examined blood samples of healthy African buffalo from South Africa, African short-horn cattle (*Bos indicus*) from Tanzania, blesbok (*Damaliscus pygargus*) from Swaziland and blue wildebeest (*Connochaetes taurinus*), klipspringer (*Oreotragus oreotragus*) and reedbuck (*Redunca arundinum*) from South Africa using RLB. A *Theileria* species similar to that discovered in sable and roan antelope was identified in these samples (Nijhof *et al.* 2005). This *Theileria* species was also isolated from a red hartebeest (*Alcelaphus buselaphus caama*) in Namibia (Spitalska, Riddell, Heyne & Sparagano 2005). This indicates a wide distribution of *Theileria* sp. (sable) throughout several different bovid species and throughout a large region of the African continent. A parasite closely related to *Theileria* sp. (sable) was also recently discovered in dogs in South Africa (Matjila, Leisewitz, Oosthuizen, Jongejan & Penzhorn 2008).

Four black rhinoceros (*Diceros bicornis*) died, presumably due to the involvement of clinical babesiosis (Nijhof, Penzhorn, Lynen, Mollel, Morkel, Bekker & Jongejan 2003). Two of these four animals had been translocated a short while before they died. The parasite was also found in five of 11 blood samples from healthy black rhinoceros, which were examined by RLB. Sequence analysis of the 18S rRNA gene confirmed that this was a new species of *Babesia*. It was named *B. bicornis*. The same authors also identified a second new parasite. The phylogenetic analysis placed this parasite within the cluster of *Theileria equi* and *Theileria youngi*. It was named *T. bicornis*. One of the individuals examined had a dual

infection with *B. bicornis* and *T. bicornis*. So far there is no evidence for the pathogenicity of *T. bicornis* in black rhinoceros (Nijhof *et al.* 2003).

In a survey carried out in Botswana, Carmichael and Hobday (1975) used blood smears of various wild animals to identify haemoparasites. *Theileria* piroplasms were found in 16.3 % of buffalo, three of ten blue wildebeest, eight of 18 tsessebe (*Damaliscus lunatus*), one of 13 lechwe (*Kobus lechwe*), six of 23 impala (*Aepyceros melampus*), four of 11 sable antelope, nine of 16 greater kudu (*Tragelaphus strepsiceros*) and in one eland. *Babesia* piroplasms were found in one blue wildebeest and one tsessebe. All animals were asymptomatic, but for one impala which was anaemic, possibly due to the parasitaemia (Carmichael & Hobday 1975).

Theilerial parasites detected in an immature impala in Kenya were blood-transmissible to other impala, but not to a steer (Grootenhuis, Young, Kimber & Drevemo 1975).

Greater kudu possibly succumbed to theilerial infection after translocation from the Eastern Cape Province to the Western Cape Province, South Africa. A new *Theileria* species was identified from blood samples of these animals using 18S rRNA gene sequence analysis, and described as *Theileria* sp. (kudu) (Nijhof *et al.* 2005). The same study also revealed that the death of a grey duiker (*Sylvicapra grimmia*) on a farm in the Gauteng Province was due to theileriosis. Using 18S rRNA gene sequence analysis, it was described as *Theileria* sp. (duiker) (Nijhof *et al.* 2005). Piroplasmosis in a grey duiker was also described by Neitz & Thomas (1948) who named the parasite *Cytauxzoon sylvicaprae*.

The case of a three-week-old tsessebe calf in the Warmbaths (now Bela Bela) district of South Africa was described by Jardine (1992). The calf had died and the blood smear showed *Theileria*-like parasites inside the red blood cells.

A male giraffe died four months after having been translocated from Namibia to Zululand. His two female companions were not affected. Post mortem findings were described by McCully, Keep & Basson (1970). The animal was anaemic and had marked haemoglobinuria. The most significant lesions were disseminated foci of haemorrhages and necrosis, especially in liver, spleen and abdomen. Very large cells heavily parasitized by schizonts were encountered in these lesions. The diagnosis of cytauxzoonosis was made in this giraffe based on the presence of schizogony in the Kupffer cells and hepatocytes and the enlargement of these parasitized cells with their tendency to become multinuclear and form syncytia. The diagnosis was also based on the presence of small erythrocytic piroplasms, which revealed some evidence of division into four (McCully *et al.* 1970).

Neitz (1931) reported the only survey of blood parasites of game in Zululand. Blood smears taken from various species were examined for haemoparasites. Piroplasms could be found in 41 of 127 zebra, 28 of 60 bushbuck (*Tragelaphus scriptus*), 27 of 50 grey duiker, 15 of 49 common reedbuck, four of 18 mountain reedbuck (*Redunca fulvorufula*), three of eight greater kudu, none of 56 warthogs (*Phacochoerus aethiopicus*), one of two steenbok (*Raphicerus campestris*), five of 40 blue wildebeest, 11 of 23 waterbuck (*Kobus ellipsiprymnus*) as well as in the single antbear (*Orycteropus afer*) examined. The author also examined one nyala (*Tragelaphus angasii*), one red duiker (*Cephalophus natalensis*), one otter (*Aonyx capensis*) and one crocodile (*Crocodylus niloticus*), all of which turned out negative for the presence of piroplasms.

Babesia and *Theileria*, were found in the blood smear of a dead bushbuck from Hluhluwe Game Reserve (Bigalke, Keep & Schoeman 1972).

It is obvious that many cases of fatal piroplasmosis in wildlife stated in the literature were connected to translocations – such as in the case of the greater kudu, which were translocated from the Eastern Cape Province to the Western Cape Province of South Africa, before they succumbed to disease (Nijhof *et al.* 2005) and in the case of two out of four black rhinoceroses (Nijhof *et al.* 2003) that presumably died due to infection with *B. bicornis*, as well as in the case of the giraffe male which died four months after translocation from Namibia to Zululand (McCully *et al.* 1970). This leads to the speculation that wild animals live in endemic stability with many piroplasms. But due to stress of translocation or other stress such as nutritional stress or pregnancy, the immune system of wild animals can be weakened and theilerial parasites, which had been present all along, suddenly caused disease (Nijhof *et al.* 2005; Penzhorn 2005).

On the other hand, it could indicate that piroplasms have a restricted geographic distribution. After translocation, animals were exposed to a piroplasm, which they had not been exposed to as young animals and against which they had not built up immunity (McCully *et al.* 1970). A good example for this scenario is also the case of nine adult sable antelope, which were exported from a German zoo to a game ranch in South Africa. The animals were kept in pens after their arrival. A sable antelope originating from South Africa had inhabited the same pen before and no tick control measures were implemented. Two of the imported animals died two months after arrival. Blood smears showed infection with *Babesia*, presumably with *B. irvinesmithi*. The other animals were prophylactically treated with imidocarb and survived (McInnes *et al.* 1991; Penzhorn 2006). This would lead to the speculation that the sable from Germany were naïve to *B. irvinesmithi* and that ticks from the South African sable antelope held in the same pen previously possibly infected the imported sable antelope (McInnes *et al.* 1991). Had it been known that *B. irvinesmithi* would pose a threat to the imported sable

antelope, prophylactic measures could have been taken from the beginning – a vaccine could even have been developed for this purpose (Penzhorn 2006).

2.2. EHRLICHIA SPECIES

There are many different *Ehrlichia* species, most of which are not pathogenic. However, others are the cause of disease in livestock, dogs and humans.

Ehrlichia ruminantium is of special importance in this respect, as it is the causative organism of heartwater, a fatal disease of domestic ruminants. In domestic ruminants, high fever, nervous signs, hydropericardium, hydrothorax, oedema of the lungs and the brain, finally leading to death of the animal, typically characterize the disease. It is one of the major causes of losses of livestock in sub-Saharan Africa (Allsopp, Bezuidenhout & Prozesky 2005). Heartwater can occur wherever a tick capable of transmission of *E. ruminantium* is present. The endemic area encompasses most of sub-Saharan Africa, including Madagascar and other islands. The disease was also introduced to the French Antillean islands of Guadeloupe and Antigua in the Caribbean Sea (Allsopp *et al.* 2005). The disease is absent from dry areas such as the Kalahari Desert and the dry coastal areas of Namibia and South Africa (Allsopp *et al.* 2005). The vectors of *E. ruminantium* are ticks of the genus *Amblyomma*: *Amblyomma hebraeum* is the main vector in South Africa while *Amblyomma variegatum* is another important vector in sub-Saharan Africa. Of lesser importance as vectors are other *Amblyomma* species such as *A. marmoreum*, *A. sparsum*, *A. pomposum*, *A. lepidum*, *A. cohaerens* and *A. gemma* (Allsopp *et al.* 2005). *Amblyomma* species native to the USA are also capable of transmitting the disease, which currently does not occur in the USA. Therefore the importation of animals carrying *E. ruminantium* into the USA could potentially lead to a heartwater disease outbreak with massive losses of naïve livestock and game as well as trade restrictions. Apparently healthy ruminant hosts have been shown to remain infective to ticks for a long period of time after infection – 361 days in cattle (Allsopp *et al.* 2005).

Infection with *E. ruminantium* has so far been proven in twelve African ruminants, three non-African ruminants and two African rodents (Peter *et al.* 2002). According to these authors, the information on host range in many reports on *E. ruminantium* infections in wild animals is compromised by lack of conclusive diagnosis and lack of supportive clinical and epidemiological data. African wild ruminants that were proven to be susceptible to *E. ruminantium* are the African buffalo, black wildebeest, blesbuck, blue wildebeest, eland, giraffe, greater kudu, sable antelope, lechwe (*Kobus leche kafuensis*), sitatunga (*Tragelaphus spekei*), springbok (*Antidorcas marsupialis*) and steenbok (Peter *et al.* 2002).

Eland (Young & Basson 1973), African buffalo (Pfitzer, Last & De Waal 2004), steenbok (Jackson & Andrew 1994) and springbok (Neitz 1944) as well as lechwe (Pandey, Minyoi, Hasebe & Mwase 1986) and sitatunga (Okoh, Oyetunde & Ibu 1986) were found dead with typical lesions indicative of heartwater.

Giraffe, eland, kudu and blue wildebeest were infected with *E. ruminantium* by infected ticks, as well as by inoculation of infected cell cultures containing *E. ruminantium* (Peter, Anderson, Burrige & Mahan 1998). The eland seroconverted after infection and ticks fed on the infected eland for up to 128 days post infection could transmit the disease to small ruminants. None of the eland showed clinical signs of disease. All blue wildebeest had seroconverted by day 128 post infection. Intrastadial transmission using ticks combined from all four wildebeest was still successful and the recipient goat died of heartwater. Intrastadial transmission by ticks from the giraffe and kudu was possible at day 85 and day 24 post infection, respectively.

Neitz (1935) infected blesbok (*Damaliscus albifrons*) as well as black wildebeest (*Conochaetus gnou*) with *E. ruminantium*. One splenectomised blesbok died 21 days after infection with pathology typical for heartwater. Sheep inoculated with blood from this blesbok also died of heartwater (Neitz 1935). Black wildebeest inoculated with *E. ruminantium* did not show any symptoms of heartwater, but sheep inoculated with the blood of the black wildebeest 13 to 30 days post infection died of heartwater.

Impala, tsessebe and sable antelope were inoculated with infected cell culture material containing *E. ruminantium* (Peter, Anderson, Burrige, Perry & Mahan 1999). Seroconversion was demonstrated in the sable and the tsessebe but not in the impala. While none of the wild animals showed any signs of disease, it was demonstrated that sable could be carriers of *E. ruminantium*, as ticks feeding on the infected animals became infected with the organisms.

Andrew and Norval (1989) infected buffalo with isolates of heartwater. The buffalo did not show any febrile reaction. *Amblyomma hebraeum* ticks were fed on these infected buffalo and then placed on heartwater-susceptible sheep. Heartwater was transmitted using ticks fed on infected buffalo for up to 161 days post infection. Buffalo from the Kruger National Park were also carriers of *E. ruminantium* (Allsopp, Theron, Coetzee, Dunsterville & Allsopp 1999).

Four impala, three blue wildebeest, one buffalo, one giraffe, one warthog and one kudu were infected with heartwater blood. Temperatures of the animals were monitored for 35 days after infection. None of the wild animals developed a febrile reaction or any signs of disease during this trial. A sheep infected with the same blood died of heartwater (Gradwell, Van Niekerk & Joubert 1976).

While rhinoceros have never been reported to show clinical signs of heartwater, Kock, Jongejan, Kock and Morkel (1992) demonstrated antibodies to *E. ruminantium* in the blood of black and white rhinoceroses. These findings indicate the possible role that rhinoceroses might play as a reservoir in the epidemiology of heartwater.

Helmeted guineafowl (*Numida meleagris*), leopard tortoise (*Geochelone pardalis*) and scrub hare (*Lepus saxitilis*) have also been proven to harbour *E. ruminantium* after artificial infection. Additionally the multimammate mouse (*Mastomys coucha*) and the striped mouse (*Rhabdomys pumilio*) are susceptible to infection, although they are unlikely to play a role in the epidemiology of the disease (Allsopp *et al.* 2005).

Fatal heartwater has also been demonstrated in several non-African ruminants such as the white-tailed-deer (*Odocoileus virginianus*), Timor deer (*Cervus timorensis*) and in chital (*Axis axis*) (Peter *et al.* 2002). BurrIDGE (1997) pointed out that the importation of wild African ruminants could pose a severe threat to the American deer and domestic ruminant population.

In South Africa, one to seven per cent of *A. hebraeum* ticks in endemic areas are infected with *E. ruminantium*. Higher rates of infection were reported from *Amblyomma* ticks in Zimbabwe (Allsopp *et al.* 2005).

It has been proven that wildlife can sustain *Amblyomma* tick populations and therefore are capable of maintaining a cycle of *E. ruminantium* transmission independently of domestic ruminants (Peter, Bryson, Perry, O'Callaghan, Medley, Smith, Mlambo, Horak, BurrIDGE & Mahan 1999). Therefore Peter *et al.* (2002) suggested in their review "further studies on the susceptibility of wild animals to *E. ruminantium* are required and should target species from heartwater-endemic areas in addition to potential hosts in heartwater-free regions." It was also pointed out that molecular-based detection assays promise to be valuable tools in this respect.

Some *Ehrlichia* species of lesser economic importance are *E. chaffeensis*, which can cause disease in humans, *E. canis*, the cause of canine ehrlichiosis, *E. bovis*, the cause of Nofel in West Africa, *E. ovina*, which infects sheep and possibly is of low economic importance (Sumption & Scott 2005). *Ehrlichia* sp. Omatjenne was described by Du Plessis (1990) and is a non-pathogenic genotype of *E. ruminantium* obtained from a *Hyalomma truncatum* tick in a heartwater-free area of Namibia.

2. 3. ANAPLASMA SPECIES

Anaplasmosis is an arthropod-borne haemoparasitic disease of cattle, which is also known as gallsickness. The disease is characterized by fever and progressive anaemia as well as icterus (Potgieter & Stoltz 2005). In southern Africa two species of *Anaplasma* are known to infect cattle – *Anaplasma marginale* and *Anaplasma centrale*. In contrast to *A. marginale*, *A. centrale* usually produces only mild disease; there is partial cross immunity between the two species (Potgieter & Stoltz 2005).

Anaplasmosis has a worldwide distribution, which is still spreading. It is endemic in most cattle-farming areas of southern Africa. In South Africa, the role that specific ticks play in the transmission of the disease has not been extensively studied. *Rhipicephalus (Boophilus) decoloratus* has been incriminated as being the most important vector. However, other tick species such as *Rhipicephalus (Boophilus) microplus*, *R. simus*, *R. evertsi evertsi* and *Hyalomma marginatum rufipes* have been shown experimentally to also be capable of transmitting the disease (Potgieter & Stoltz 2005). In addition, anaplasmosis is also easily transmitted mechanically by needle passage of infected blood and by blood-sucking arthropods (Potgieter & Stoltz 2005). Cattle reared in endemic areas usually develop a naturally acquired immunity to the disease.

Anaplasmosis in sheep and goats, caused by *A. ovis* and *A. mesaeorum*, is similar to gallsickness but usually subclinical and mild. Recovered animals remain carriers of the organisms. Ovine and caprine anaplasmosis occurs in many parts of the world but is not perceived to be of high economic importance (Stoltz 2005).

It has been shown that blesbok, grey duiker and black wildebeest are susceptible to experimental infection with *A. marginale* but infections are subclinical (Neitz & Du Toit 1932; Potgieter & Stoltz 2005). One blue wildebeest developed parasitaemia of *A. marginale* after splenectomy (BurrIDGE, 1975). Blesbok are also susceptible to infection with *A. centrale* (Neitz & Du Toit 1932).

Anaplasma species have also been recorded in giraffe, sable antelope, buffalo and black wildebeest (Potgieter & Stoltz 2005). Thomas *et al.* (1982) examined the blood smears of 124 sable antelopes from South Africa and Zimbabwe. Only one of these smears was positive for an *Anaplasma* species. The parasitaemia was less than 1%.

Carmichael & Hobday (1975) examined blood smears of 282 wild bovids from Botswana, including 190 buffalo, 23 impala, ten blue wildebeest, 18 tsessebe, one eland, 13 lechwe, 16 kudu and 11 sable antelope. *Anaplasma* species were found in buffalo only, of which 27.8% were positive. Intra-erythrocytic bodies were also found in blood smears of other species.

As these phenomena could not be classified properly and were difficult to differentiate, they were therefore classified as Howell-Jolly bodies. Peirce (1972) found that *A. marginale* occurred in eland in Kenya.

Sera of antelope living on farmland where cattle are dipped frequently showed fewer positive reactions in the capillary tube agglutination and indirect fluorescent antibody test against *A. marginale* than sera of antelope grazing in the vicinity of non-dipped cattle (Löhr & Meyer 1974). It has consequently been suggested that antelope species in South Africa may be the natural hosts for the *Anaplasma* species of domestic ruminants. This theory is supported by the fact that eland can be carriers of *A. marginale* as well as of *A. ovis*, and might therefore play an important role in the epidemiology of anaplasmosis (Ngeranwa, Venter, Penzhorn, Soi, Mwanzia & Nyongesa 1998).

An epidemiological study of anaplasmosis at the wildlife–livestock interface in Kenya indicated a high seroprevalence of antibodies to *Anaplasma* species in wildlife as well as livestock populations (Ngeranwa, Shompole, Venter, Wambugu, Crafford & Penzhorn 2008). Seroprevalence of eland, blue wildebeest, kongoni (*Damaliscus korrigum*), impala, Thomson's gazelle (*Gazella thomsonii*), Grant's gazelle (*Gazella granti*), giraffe, plains zebra (*Equus quagga*), cattle, sheep and goats examined was between 75 and 100%.

There is also evidence that game might harbour *Anaplasma* species or strains different from those of domestic ruminants (Thomas *et al.* 1982). Kuttler (1984) pointed out that the epidemiologic significance of anaplasmosis in wildlife has yet to be determined. The only wild animal in which *Anaplasma* is reported to produce serious clinical disease is the giraffe (Augustyn & Bigalke 1978; Kuttler 1984).

2.4. THE IMPORTANCE OF TICK-BORNE DISEASES IN WILDLIFE

Tick-borne haemoparasitic diseases are globally the economically most important parasitic diseases (Uilenberg 1995). Therefore it would be imperative to know as much as possible about these parasites in order to be able to gain control over these diseases. In this regard it would be important to find out how readily piroplasms, *Ehrlichia* and *Anaplasma* species cross the interspecies barrier and what wildlife reservoirs exist that are of importance to the livestock industry. This is especially important with regard to *E. ruminantium* and international movement of African wild and domestic ruminants (BurrIDGE 1997). But even local movement of wild ruminants from heartwater-endemic areas that harbour *E. ruminantium* could lead to the translocation of different heartwater strains into areas where animals have not yet been exposed to those strains. As there is limited cross protection, this could lead to localized outbreaks of heartwater, especially in domestic ruminants – even in heartwater-endemic areas (Allsopp *et al.* 2005).

For conservation purposes, it would be of importance to know the abundance, epidemiology and pathogenicity of piroplasms that affect wildlife. Due to the fact that many piroplasms were isolated from healthy animals (Neitz 1931; Carmichael & Hobday 1975; Nijhof *et al.* 2003; Nijhof *et al.* 2005), it is presently believed that wild animals – similar to cattle – live in epidemic stability with piroplasms (De Vos *et al.* 2005; Lawrence *et al.* 2005a; Penzhorn 2005). Many piroplasms and haemoparasites were discovered in blood smears several years ago, when molecular typing was not yet available. It is not clear, therefore, whether piroplasms detected in wildlife are species-specific or whether just a few species of piroplasms can infect a variety of game species – such as in the case of *Theileria* sp. (sable) (Nijhof *et al.* 2005).

If endemic stability exists between wild mammals and their respective haemoparasites, this would be an important factor, which has to be taken into account during translocation of wildlife, as well as when captive-bred wildlife is reintroduced into areas. Measures could be taken accordingly to prevent unnecessary death of especially rare species such as sable, roan or black rhinoceros. Populations that are naïve to specific piroplasms must be protected from future exposure or, if viable, they should be vaccinated. Currently the infection and treatment method is the main method of vaccination against haemoparasites such as *Babesia*, *Theileria*, *Ehrlichia* and *Anaplasma* species. These live vaccines have to be specially prepared for each parasite species as there is only limited cross protection (De Vos *et al.* 2005; Lawrence *et al.* 2005a).

2.5. NYALA (*TRAGELAPHUS ANGASII*)

The nyala is a medium-sized antelope that naturally inhabits dense bush in the humid parts of southern Africa. Nyala occur as far north-east as southern Malawi, Mozambique, Zimbabwe and Swaziland. In South Africa, nyala naturally occur in northern KwaZulu-Natal, the northern Kruger National Park, along the Limpopo River valley and westwards from the Kruger National Park to the Swartwater area (Pfitzer & Kohrs 2005). While most samples for this thesis were taken from nyala within their original range, 12 specimens were from the Eastern Cape Province. The species had been introduced from northern KZN to the Eastern Cape 20 years previously. The Eastern Cape Province is not regarded as the original habitat of nyala. However, nyala introduced onto mixed bushveld of the coastal areas of the Eastern Cape Province usually are breeding up and doing very well. The farm where samples were taken was 15 km from Grahamstown and heartwater, redwater as well as anaplasmosis are known to occur in cattle in this area. *Rhipicephalus appendiculatus* is also present in this area (L. Amaral, State Veterinary Services East London, pers. comm. 2008).

Nyala ewes, lambs and subadults are bright chestnut in colour, often with a white chevron between the eyes, white spots below the eyes and numerous white vertical stripes along their

sides. White spots may also be visible on their haunches. An adult ewe weighs about 60 kg. Nyala bulls, in contrast, are much larger and weigh around 110 kg. They are very dark grey to black in colour, with faint vertical stripes along their sides. The chevron between the eyes is usually pronounced and there are white spots underneath the eyes. The haircoat is long on the underside of the neck and belly. Males also carry spiralled horns (Pfitzer & Kohrs 2005).



Figure 1: Nyala male with female and subadults

Nyala in particular are a good species to be surveyed for this purpose. This is due to the fact that nyala, especially from the Zululand area, are very effective tick carriers (Horak, Boomker & Flamand 1995). They share their bushveld and riverine habitat with kudu, bushbuck and grey duiker, from all of which piroplasms have been isolated (Neitz 1931; Nijhof *et al.* 2005). Nyala from Hluhluwe-iMfolozi Park (HiP) in KwaZulu-Natal and from Kruger National Park (KNP) were examined by Horak, Potgieter, Walker, De Vos & Boomker (1983). Two subadult nyala bulls from HiP collected in September 1978 carried a total of 6 103 and 6 771 ticks, respectively. Two adult bulls from the KNP examined in October 1981 carried a total tick burden of 1 070 ticks and 1 224 ticks, respectively. Nymphae and larvae of *Rhipicephalus* spp. made up the majority of ticks on these animals (Horak *et al.* 1983). Baker & Keep (1970) list the following tick species that have been found on nyala in KwaZulu-Natal game reserves: *Amblyomma hebraeum*, *Ixodes pilosus*, *Haemaphysalis silacea*, *Rhipicephalus appendiculatus*, *R. evertsi evertsi*, *R. maculatus*, *R. muelhensi*, *R. pravus*, *R. sanguineus*, *R. simus* and *R. (Boophilus) decoloratus*. Horak *et al.* (1983) added *Rhipicephalus zambeziensis* to the list of ticks found on nyala – these animals were from the KNP. Additionally the tick species *Haemaphysalis aciculifer* was reported from nyala at HiP (Horak *et al.* 1995).

Nyala could therefore potentially play a huge role in the spread of piroplasms and other tick-borne pathogens. Because they have been translocated so widely and mixed with other

valuable game in intensive breeding projects, they might have potentially contributed to the spread of various piroplasms, if they were carriers. Keep (1971) conducted a study on parasites and pathology of nyala culled in Zululand game reserves. In four of sixteen blood smears he could find “*Theileria*-like piroplasms”.

As so many nyala are captured and handled individually for translocation, a survey for blood parasites is comparatively easy and non-invasive to these animals. For all these reasons, nyala are an ideal species to be surveyed in the quest of finding piroplasms and other tick-borne diseases in wildlife.

2. 6. POLYMERASE CHAIN REACTION (PCR) AND REVERSE LINE BLOT (RLB) HYBRIDISATION

There are several techniques to detect haemoparasites (Figueroa & Buening 1995): The definite laboratory diagnosis of *Anaplasma*, *Babesia* and *Theileria* organisms in acutely infected animals is generally based on the microscopic examination of peripheral blood smears for the presence of intraerythrocytic or intralymphocytic bodies which could be differentiated by their morphological properties. *Ehrlichia ruminantium* organisms can be demonstrated in acutely infected animals by means of microscopic examination of a brain smear, searching for rickettsial colonies in the endothelial cells.

A characteristic feature of haemotropic diseases, however, is that animals that have recovered from an acute infection often become carriers of the respective organisms. Carrier animals serve as reservoir of infection for the tick vector and cannot be clinically differentiated from uninfected animals. Organisms are usually present in very low numbers and cannot be demonstrated by means of a traditional blood smear or brain smear method (Figueroa & Buening 1995).

There is an array of immunological methods, but these are indirect methods. Often carrier animals have very low antibody titres, leading to false negative results. In addition, serological techniques such as the immunofluorescent antibody test (IFAT) give rise to cross reactions amongst different *Theileria* species (Gubbels *et al.* 1999).

Several PCR-based diagnostic procedures have been developed for the identification of haemoparasites. Increased sensitivity and specificity can be achieved by combining PCR with a specific hybridisation by means of a reverse line blot, a macro-array that is also capable of identifying mixed infections (Garcia-Sanmartin, Nagore, Garcia-Perez, Juste & Hurtado 2006).

The RLB used in this study to identify different piroplasms was first described by Gubbels *et al.* (1999) for the simultaneous detection and identification of tick-borne parasites infecting

cattle and small ruminants. It was shown to have a detection limit of 10^{-6} % parasitaemia. The high sensitivity enables one to determine the carrier state of most parasites. The RLB was successfully used by Gubbels *et al.* (1999) to screen cattle in Spain for *Theileria* and *Babesia* organisms and to identify carrier animals, as well as mixed infections. This RLB was also successfully used by Nijhof *et al.* (2003) to identify *B. bicornis* as well as *T. bicornis* – two new species of piroplasms – in black rhinoceros. It was also used by Nijhof *et al.* (2005) to detect new *Theileria* species in four species of African antelope. Almeria, Castella, Ferrer, Gutierrez, Estrada-Peña & Sparagano (2002) used the RLB to identify piroplasms in blood samples from cattle in Minorca. Garcia-Sanmartin *et al.* (2006) used this method to detect single and mixed infections of several *Babesia* and *Theileria* species in cattle of northern Spain. Schnittger, Yin, Qi, Gubbels, Beyer, Niemann, Jongejan & Ahmed (2004) developed an RLB to detect piroplasms of small ruminants, such as *Theileria ovis*, *Theileria lestoquardi*, *Theileria separata*, *Babesia ovis* and others.

Schouls, Van de Pol, Sjoerd, Rijpkema & Schot (1999) developed an RLB to simultaneously detect different *Ehrlichia* species, as well as *Borrelia burgdorferi* and *Bartonella* species in up to 40 samples at the same time. Bekker *et al.* (2002) developed an RLB for the simultaneous detection of *Anaplasma* and *Ehrlichia* species.

The RLB described by Gubbels *et al.* (1999) and Bekker *et al.* (2002) were combined and used by Georges, Loria, Riili, Greco, Caracappa, Jongejan & Sparagano (2001) to detect *Theileria*, *Babesia*, *Anaplasma* and *Ehrlichia* species in blood samples from dairy cattle in Sicily. Oura, Bishop, Wamapande, Lubega & Tait (2004) also used the combined RLB to study haemoparasites in cattle in Uganda.

A commercially available RLB (Isogen®, Maarsen, The Netherlands) was developed that could differentiate between several *Theileria* and *Babesia* as well as *Anaplasma* and *Ehrlichia* species on the same membrane (Taoufik, Sonneveld, Nijhof, Hamidjaja, Pillay, Oosthuizen, de Boer & Jongejan 2005). This development made the RLB test more cost effective as it now allows the simultaneous analysis of multiple samples against multiple probes. It is an excellent tool for screening for singular and mixed infections of multiple haemoparasites simultaneously, especially with a large number of samples (Taoufik *et al.* 2005).

2.7. OBJECTIVES OF THIS STUDY

The objectives of this study were to screen nyala blood samples for the occurrence of tick-borne haemoparasites and to identify haemoparasites found in blood of these nyala.

3. MATERIALS AND METHODS

3.1. SAMPLE COLLECTION

Blood samples were obtained from nyala during routine capture procedures carried out in 2007 and 2008. Samples (n = 131) were collected from four private farms in the Pongola area. Of these, 30 were collected on Whatman® filter paper grade F 572-02 (Supplier: Merck). The remainder was taken as blood samples in EDTA tubes. Twelve samples were obtained in EDTA tubes from captures in the Eastern Cape Province (Figure 2).

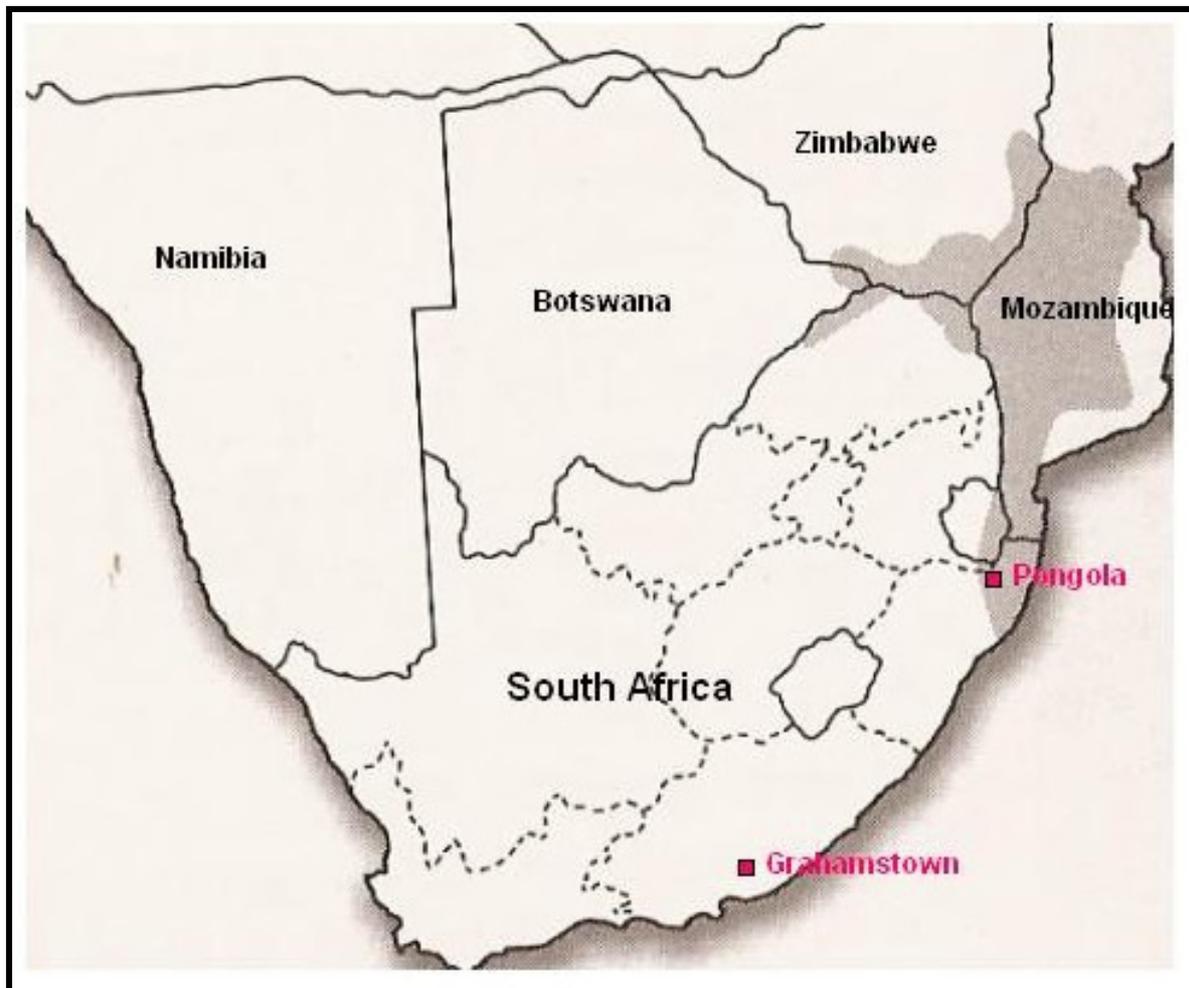


Figure 2: The natural distribution of nyala in southern Africa (grey) and sample areas (red)

Capture was carried out by means of chemical immobilisation. Drugs used for this purpose were Thiafentanyl Oxalate (A3080) and Azaperone, which were administered by the Daninject darting system – a gas-powered dart gun. Transmitter darts were used to facilitate rapid

recovery of the animals. EDTA blood samples were usually taken from immobilised nyala from the jugular or auricular veins. Vacutainer® tubes were used for this purpose. The EDTA blood was kept at about -20°C until examined in the Molecular Biology Laboratory, Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria.

Capture was also carried out passively. In this case, animals were attracted by feed and water to enter a capture boma. Once the animals were inside, the boma was closed and the animals were herded into the game trailer. After tranquillization with Haloperidol, the animals could be injected with further medication by hand and a blood sample from the ear was taken on Whatman® filter paper. The filter paper was stored in a dry, dark place.

For each sample, the month of capture, sex and age group of the animal as well as capture location was noted.

The sex ratio was about 28% males and the rest females. Captured animals were mainly adults but some subadults were also sampled.

3.2. LABORATORY PROCEDURES

Samples were analysed at the Molecular Biology Laboratory of the Department of Veterinary Tropical Diseases at the Faculty of Veterinary Science of the University of Pretoria at Onderstepoort.

3.2.1. DNA Extraction

Blood samples were aliquoted in 2 ml tubes and kept at -20°C until further processing.

DNA was extracted from 200 μl of whole blood and 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.

The success of the DNA extraction was controlled by agarose gel electrophoresis and spectrophotometric readings. Samples of each batch on which DNA extraction was carried out were tested sporadically.

3.2.2. Polymerase Chain Reaction (PCR)

The PCR was conducted as described by Nijhof *et al.* (2003) and Nijhof *et al.* (2005). 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, 2.5 µl of DNA to a total volume of 25 µl. In the case of DNA extracted from blood spotted on filter paper, 5 µl of DNA was used. 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 as shown in Table 1.

Table 1: PCR thermocycler programme

Number of cycles	Duration	Temperature in °C
1	3 min	37
1	10 min	94
2	20 sec	94
	30 sec	67
	30 sec	72
2	20 sec	94
	30 sec	65
	30 sec	72
2	20 sec	94
	30 sec	63
	30 sec	72
2	20 sec	94
	30 sec	61
	30 sec	72

2	20 sec	94
	30 sec	59
	30 sec	72
40	20 sec	94
	30 sec	57
	30 sec	72
1	7 min	72
Final extension		4

The PCR amplicons were verified in samples of each batch of PCR analysis using agarose gel electrophoresis before it was analysed by RLB hybridisation. Five microlitres of PCR product were mixed with 3 µl of loading dye (Inqaba Biotechnology, South Africa) for this purpose and aliquoted into the wells of the gel. A 100 bp ladder was used.

3.2.3. Reverse line blot (RLB) hybridization assay

The PCR products were analysed using the RLB hybridisation technique, first described by 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).

3.2.3.1 Preparation of membrane:

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 Isogen membrane are listed in Table 2. For preparation of the in-house membrane, the species-specific oligonucleotides were diluted in 150 µl 0.5 M NaHCO₃. The membrane was then marked and incubated for 10 min in 16% EDAC at room temperature. It was then rinsed with demineralised water. Genus- and species-specific probes included on this membrane are listed in Table 3.

Table 2: Species-specific probes on the commercial TBD-RLB kit (Isogen Life Science, the Netherlands). Differences in probes of the two membranes are highlighted.

Lane	Species	Probe Sequence from 5' to 3'
1	<i>Ehrlichia</i> / <i>Anaplasma</i> catch-all	GGG GGA AAG ATT TAT CGC TA
2	<i>Anaplasma centrale</i>	TCG AAC GGA CCA TAC GC
3	<i>Anaplasma marginale</i>	GAC CGT ATA CGC AGC TTG
4	<i>Anaplasma phagocytophilum</i>	TTG CTA TAA AGA ATA ATT AGT GG
5	<i>Anaplasma phagocytophilum</i>	TTG CTA TGA AGA ATA ATT AGT GG



6	<i>Anaplasma phagocytophilum</i>	TTG CTA TAA AGA ATA GTT AGT GG
7	<i>Anaplasma phagocytophilum</i>	TTG CTA TAG AGA ATA GTT AGT GG
8	<i>Ehrlichia ruminantium</i>	AGT ATC TGT TAG TGG CAG
9	<i>Anaplasma bovis</i>	GTA GCT TGC TAT GRG AAC A
10	<i>Ehrlichia chaffeensis</i>	ACC TTT TGG TTA TAA ATA ATT GTT
11	<i>Ehrlichia</i> sp. Omatjenne	CGG ATT TTT ATC ATA GCT TGC
12	<i>Ehrlichia canis</i>	TCT GGC TAT AGG AAA TTG TTA
13	<i>Theileria / Babesia</i> catch-all	TAA TGG TTA ATA GGA RCR GTT G
14	<i>Babesia felis</i>	TTA TGC GTT TTC CGA CTG GC
15	<i>Babesia divergens</i>	ACT RAT GTC GAG ATT GCA C
16	<i>Babesia microti</i>	GRC TTG GCA TCW TCT GGA
17	<i>Babesia bigemina</i>	CGT TTT TTC CCT TTT GTT GG
18	<i>Babesia bovis</i>	CAG GTT TCG CCT GTA TAA TTG AG
19	<i>Babesia rossi</i>	CGG TTT GTT GCC TTT GTG
20	<i>Babesia canis canis</i>	TGC GTT GAC GGT TTG AC
21	<i>Babesia canis vogeli</i>	AGC GTG TTC GAG TTT GCC
22	<i>Babesia major</i>	TCC GAC TTT GGT TGG TGT
23	<i>Babesia bicornis</i>	TTG GTA AAT CGC CTT GGT C
24	<i>Babesia caballi</i>	GTT GCG TTK TTC TTG CTT TT
25	<i>Theileria</i> sp. (kudu)	CTG CAT TGT TTC TTT CCT TTG
26	<i>Theileria</i> sp. (sable)	GCT GCA TTG CCT TTT CTC C
27	<i>Theileria bicornis</i>	GCG TTG TGG CTT TTT TCT G
28	<i>Theileria annulata</i>	CCT CTG GGG TCT GTG CA
29	<i>Theileria buffeli</i>	GGC TTA TTT CGG WTT GAT TTT
30	<i>Theileria</i> sp. (buffalo)	CAG ACG GAG TTT ACT TTG T
31	<i>Theileria mutans</i>	CTT GCG TCT CCG AAT GTT
32	<i>Theileria parva</i>	GGA CGG AGT TCG CTT TG
33	<i>Theileria taurotragi</i>	TCT TGG CAC GTG GCT TTT
34	<i>Theileria velifera</i>	CCT ATT CTC CTT TAC GAG T
35	<i>Theileria equi</i>	TTC GTT GAC TGC GYT TGG
36	<i>Theileria lestoquardi</i>	CTT GTG TCC CTC CGG G

(Symbols indicate degenerate positions: R = A/G, W = A/T, K = G/T)

Table 3: Genus- and species-specific probes present on the in-house prepared membrane. Differences in probes of the two membranes are highlighted.

Lane	Species	Probe Sequence from 5' to 3'
1	INK	
2	<i>Ehrlichia / Anaplasma</i> catch-all	GGG GGA AAG ATT TAT CGC TA
3	<i>Anaplasma centrale</i>	TCG AAC GGA CCA TAC GC
4	<i>Anaplasma marginale</i>	GAC CGT ATA CGC AGC TTG
5	<i>Anaplasma phagocytophilum</i>	TTG CTA TAA AGA ATA ATT AGT GG
6	<i>Ehrlichia ruminantium</i>	AGT ATC TGT TAG TGG CAG
7	<i>Anaplasma bovis</i>	GTA GCT TGC TAT GRG AAC A
8	<i>Ehrlichia chaffeensis</i>	ACC TTT TGG TTA TAA ATA ATT GTT
9	<i>Ehrlichia</i> sp. Omatjenne	CGG ATT TTT ATC ATA GCT TGC
10	<i>Ehrlichia canis</i>	TCT GGC TAT AGG AAA TTG TTA
11	<i>Theileria / Babesia</i> catch-all	TAA TGG TTA ATA GGA RCR GTT G
12	<i>Theileria</i> catch-all	ATT AGA GTG CTC AAA GCA GGC
13	<i>Babesia</i> catch-all 1	ATT AGA GTG TTT CAA ACA GGC
14	<i>Babesia</i> catch-all 2	ACT AGA GTG TTT CAA ACA GGC
15	<i>Babesia felis</i>	TTA TGC GTT TTC CGA CTG GC
16	<i>Babesia divergens</i>	ACT RAT GTC GAG ATT GCA C
17	<i>Babesia microti</i>	GRC TTG GCA TCW TCT GGA
18	<i>Babesia bigemina</i>	CGT TTT TTC CCT TTT GTT GG
19	<i>Babesia bovis</i>	CAG GTT TCG CCT GTA TAA TTG AG
20	<i>Babesia rossi</i>	CGG TTT GTT GCC TTT GTG
21	<i>Babesia canis canis</i>	TGC GTT GAC GGT TTG AC
22	<i>Babesia canis vogeli</i>	AGC GTG TTC GAG TTT GCC
23	<i>Babesia major</i>	TCC GAC TTT GGT TGG TGT
24	<i>Babesia bicornis</i>	TTG GTA AAT CGC CTT GGT C
25	<i>Babesia caballi</i>	GTT GCG TTK TTC TTG CTT TT
26	<i>Theileria</i> sp. (kudu)	CTG CAT TGT TTC TTT CCT TTG
27	<i>Theileria</i> sp. (sable)	GCT GCA TTG CCT TTT CTC C
28	<i>Theileria bicornis</i>	GCG TTG TGG CTT TTT TCT G
29	<i>Theileria annulata</i>	CCT CTG GGG TCT GTG CA
30	<i>Theileria buffeli</i>	GGC TTA TTT CGG WTT GAT TTT
31	<i>Theileria</i> sp. (buffalo)	CAG ACG GAG TTT ACT TTG T
32	<i>Theileria mutans</i>	CTT GCG TCT CCG AAT GTT
33	<i>Theileria parva</i>	GGA CGG AGT TCG CTT TG
34	<i>Theileria taurotragi</i>	TCT TGG CAC GTG GCT TTT

35	<i>Theileria velifera</i>	CCT ATT CTC CTT TAC GAG T
36	<i>Theileria equi</i>	TTC GTT GAC TGC GYT TGG
37	<i>Theileria lestoquardi</i>	CTT GTG TCC CTC CGG G
38	<i>Theileria separata</i>	GGT CGT GGT TTT CCT CGT
39	<i>Theileria ovis</i>	TTG CTT TTG CTC CTT TAC GAG
40	<i>Babesia sable</i>	GCT GCA TTG CCT TTT CTC C
41	<i>Babesia gibsoni</i>	CAT CCC TCT GGT TAA TTT G
42	INK	

(Symbols indicate degenerate positions: R = A/G, W = A/T, K = G/T)

3.2.3.2 RLB hybridization:

The membrane was incubated for 5 min in 10 ml 2 x SSPE / 0.1% SDS at room temperature. Twenty microlitres of PCR product (comprising of 10 µl of PCR product obtained with the *Theileria* / *Babesia* primers and 10 µl of PCR product obtained with the *Ehrlichia* / *Anaplasma* primers) was diluted in 140 µl 2 x SSPE / 0.1% SDS. The diluted PCR product was denatured for 10 min at 100°C in one of the thermocyclers and then immediately cooled on ice.

The membrane was placed in the miniblotter with slots perpendicular to the line pattern of the applied probes. The ink-lane was aligned to be directly under the opening of the slots. All residual fluid was removed by aspiration with a vacuum pump. Slots were then filled with the diluted PCR product and empty slots were filled with buffer to avoid cross flow. Hybridization took place at 42°C for 60 min on a horizontal surface. Afterwards, samples were removed by aspiration and the membrane was removed from the miniblotter. The membrane was washed twice in preheated 2 x SSPE / 0.5% SDS for 10 min at 50°C in a shaking incubator using gentle shaking. The membrane was then incubated with 100 ml 2 x SSPE / 0.5% SDS + 25 µl streptavidin-POD (peroxidase-labelled) conjugate (Roche Diagnostics) (1.25 U) for 30 min at 42°C. The membrane was then washed twice in preheated 2 x SSPE / 0.5% SDS for 10 min at 42°C in a shaking incubator using gentle shaking. The membrane was then washed twice with 2 x SSPE for 5 min at room temperature using gentle shaking. Ten millilitres ECL solution (5 ml ECL 1 + 5 ml ECL 2) (Perkin Elmer) were spread over the membrane in a plastic container and it was incubated for 1 min at room temperature. The membrane was placed between two clean overhead sheets into an exposure cassette. The X-ray film was exposed for 10 min and the X-ray film was developed for detection of hybridized PCR products, which were visualized by chemiluminescence. The film was placed in a grid and each sample lane correlated with the DNA probes.

After use, the RLB membrane was stripped according to instructions for care and maintenance of the membrane. This procedure included two washes with 1% SDS, preheated to 75°C and one wash with 20mM EDTA at room temperature. The membrane was stored at 4°C in 20 mM EDTA, pH 8.

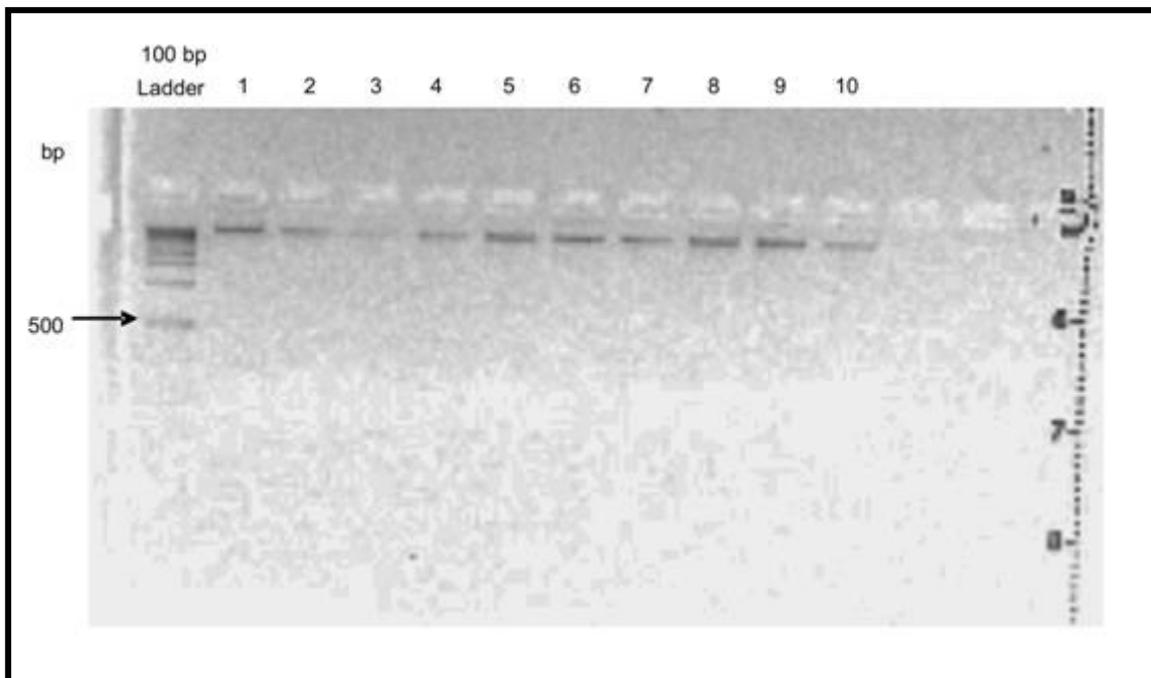
4. RESULTS

A total of 143 blood specimens were collected from nyala during 2007 and 2008. The majority (n = 101) were specimens from northern KZN collected in EDTA. A further 30 specimens from the same general area were collected on filter paper. Twelve specimens in EDTA were collected from a game ranch in the Eastern Cape.

4.1. DNA EXTRACTION

DNA was successfully extracted from all samples. Random testing of various samples of different batches validated this. For this purpose spectrophotometry as well as agarose gel electrophoresis were used. The DNA concentration of 10 representative samples was spectrophotometrically measured after extraction; the concentration ranged from 8.32 to 57.2 ug/ml.

The agarose gel electrophoresis of these samples is shown in figure 3. It must be kept in mind that total DNA is measured with these procedures. Total DNA in blood mainly stems from the animal, any biological contamination, as well as DNA from haemoparasites.



**Figure 3: Agarose gel electrophoresis of a subset of the genomic DNA preparations.
Lane 1 = 100 bp ladder.**

4.2. PCR AMPLIFICATION

To test if the PCR amplification was successful, amplicons of randomly selected samples were subjected to agarose gel electrophoresis. The V4 hypervariable region of the 18S rRNA gene was successfully amplified using the *Theileria* and *Babesia* genus-specific primers, as evident by a 500 bp amplicon seen on an agarose gel (Figure 4). Similarly, the V1 hypervariable region of the 16S rRNA gene using the *Ehrlichia* and *Anaplasma* genus-specific primers was successfully amplified (Figure 4).

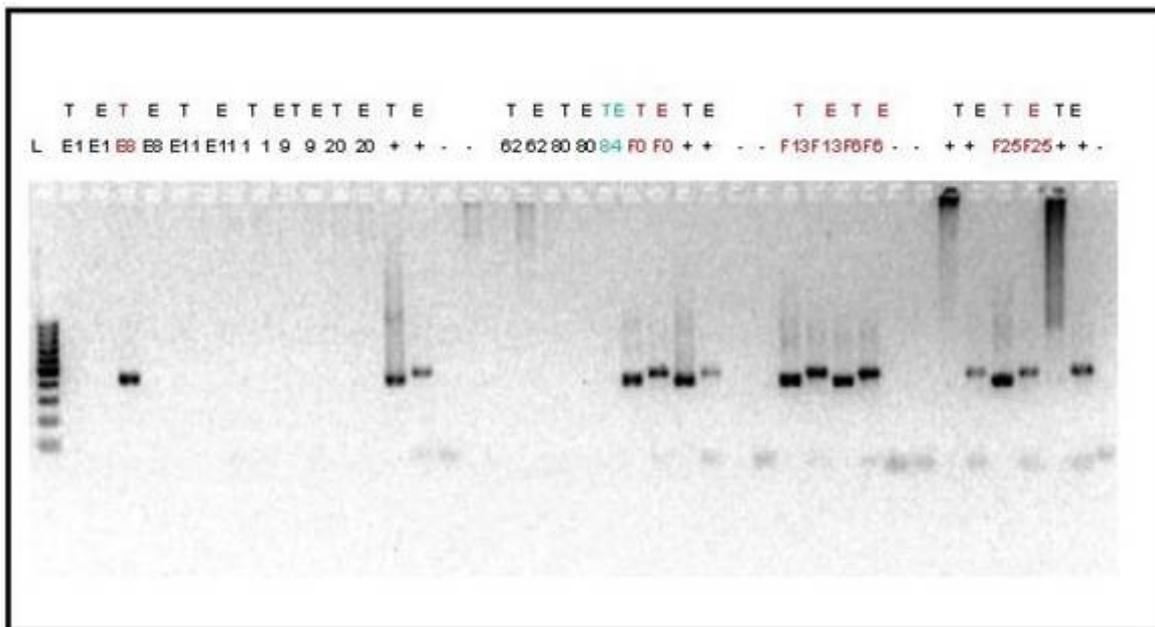


Figure 4: Agarose gel electrophoresis demonstrating the successful amplification of some of the PCR products with *Theileria* / *Babesia* (marked as T) and *Ehrlichia* / *Anaplasma* (marked as E) primers. The ladder used was a 100 bp ladder and it was added to the first well. Samples marked + and – were PCR controls. Five microlitres of PCR product was mixed with 3 µl of dye and added into each well for this test. Note that sample 84 (marked green) was a pipetting error – both the *Theileria* and *Ehrlichia* amplified products went into the same well.

4.3. RLB RESULTS

4.3.1. *Babesia* and *Theileria*

In total, 28 of the 143 samples (20%) subjected to PCR amplification and RLB hybridization tested positive for the presence of piroplasms (Table 4). Interestingly, 27 of the 28 positive samples were originally collected on filter paper. With the exception of one specimen from the Eastern Cape (E8), all EDTA blood samples collected (n = 113) were found to be apparently

free of piroplasms. Sample E8 tested positive for the presence of *Theileria* sp. (kudu), *T. bicornis*, *T. buffeli* and *T. taurotragi*.

The most common haemoparasites found in nyala blood sampled on filter paper were *Theileria* sp. (kudu) and *T. buffeli*, both of which were carried by 22 animals, always as mixed infections.

Two animals from KZN (samples F0 and F1) were positive for the presence of *T. bicornis* and one animal for *Theileria* sp. (sable). The RLB hybridization signal for *Theileria* sp. (sable) was very weak, however.

Only the animal from the Eastern Cape (E8) tested positive for the presence of *T. taurotragi*.

None of the sampled animals carried *Babesia* species.

On five occasions PCR products did not hybridize with any of the *Babesia* or *Theileria* species-specific probes, and only hybridized with the *Babesia* / *Theileria* genus-specific probe suggesting the presence of a novel species or variant of a species. In all five cases, these genus-specific signals were weak.

4.3.2 Ehrlichia and Anaplasma

Twelve animals tested positive for the presence of *Ehrlichia* sp. Omatjenne, an apathogenic species. This was the second-most common infection. Eleven of the 12 animals carried this pathogen together with other haemoparasites. No animal tested positive for the presence of *E. ruminantium*.

Five animals tested positive for the presence of *Anaplasma marginale*, three of which were also positive for *Ehrlichia* sp. Omatjenne. All of the carriers of *A. marginale* were also infected with *Theileria* species. Only one sample (F15) tested positive for the presence of *A. bovis*.

Anaplasma centrale could not be detected in any of the nyala.

In seven of the samples, PCR products failed to hybridize with any of the *Ehrlichia* or *Anaplasma* species-specific probes, and only hybridized with the *Ehrlichia* / *Anaplasma* genus-specific probe suggesting the presence of a novel species or variant of a species. These signals were, however, very often weak and hardly visible.

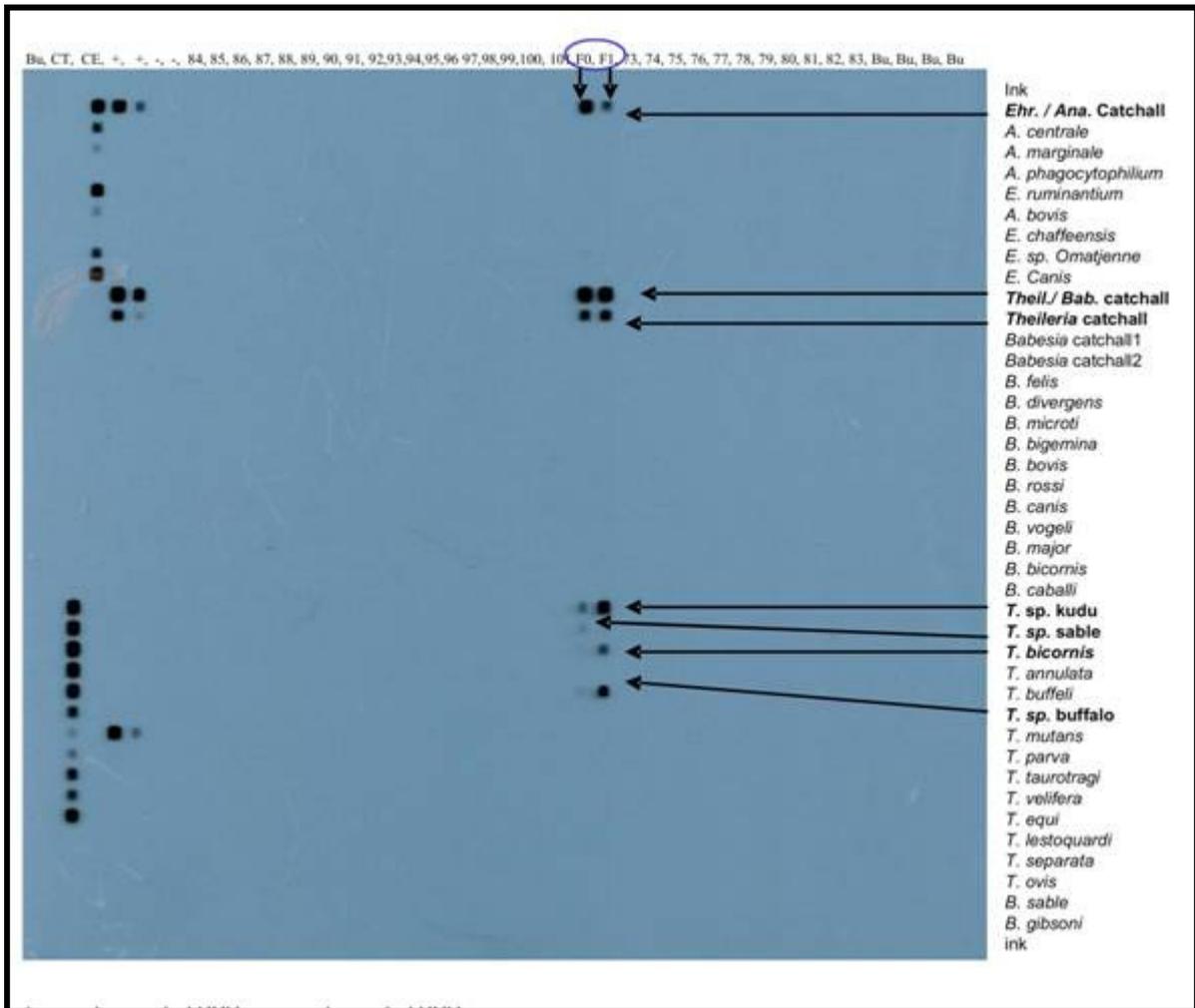


Figure 5: RLB results of some of the nyala specimens investigated. Species-specific oligonucleotides are applied in horizontal lanes, and PCR products in vertical lanes. *Theileria / Babesia* (CT), *Ehrlichia / Anaplasma* (CE) control samples are in lanes 2 and 3. The positive PCR controls are in line 4 and 5 and negative PCR controls in line 5 and 6.

4.3.3. Summary of results

The results are summarised in Table 4 and Figure 6. Blood samples in EDTA from the Pongola area (KZN) all tested negative (1 to 101) and are not listed in this table. In contrast, most of the 30 samples from the same area that were collected on filter paper (F0 to F29), showed positive results and are listed in this summary. The only negative samples were sample F2, F3 and F11, which are therefore not listed in Table 4. Only one (E8) of the 12 EDTA blood samples from the Eastern Cape Province (E1 to E12) tested positive and is listed in this summary.

Positive results came from each of the farms where filter paper samples of nyala were taken. As many more females were captured and tested than males, the sex ratio in the test results merely indicates that males as well as females can be infected. Some of the infected animals were animals of less than 6 months of age; sample F8 was from a hand-raised male of about two months of age.

Table 4: Summary of RLB results. Negative results were either true negatives or below detection limit of the test.

Sample number	Farm	Sex	RLB result			
			<i>Theileria/Babesia</i> genus specific result	<i>Theileria/Babesia</i> species-specific result	<i>Ehrlichia/Anaplasma</i> genus specific result	<i>Ehrlichia/Anaplasma</i> species-specific result
F0	Farm 1, Pongola, KZN	M	+	<i>Theileria</i> sp. (kudu), <i>T. bicornis</i> , <i>T. buffeli</i> , <i>Theileria</i> sp. (<i>sable</i>)	+	Negative
F1	Farm 1, Pongola, KZN	F	+	<i>Theileria</i> sp. (kudu), <i>T. bicornis</i> , <i>T. buffeli</i>	+	Negative
F4	Farm 1, Pongola, KZN	F	+	<i>Theileria</i> sp. (kudu), <i>T. buffeli</i>	Negative	
F5	Farm 1, Pongola, KZN	F	+	Negative	+	Negative
F6	Farm 1, Pongola, KZN	M	+	<i>Theileria</i> sp. (kudu), <i>T. buffeli</i>	+	<i>Ehrlichia</i> sp. Omatjenne
F7	Farm 1, Pongola, KZN	F	Negative	Negative	+	<i>Ehrlichia</i> sp. Omatjenne
F8	Farm 2, Pongola, KZN	M	+	Negative	Negative	Negative
F9	Farm 1, Pongola, KZN	F	+	<i>Theileria</i> sp. (kudu), <i>T. buffeli</i>	+	<i>Ehrlichia</i> sp. Omatjenne, <i>Anaplasma marginale</i>
F10	Farm 1, Pongola, KZN	F	+	<i>Theileria</i> sp. (kudu), <i>T. buffeli</i>	Negative	Negative
F12	Farm 1, Pongola, KZN	F	+	<i>Theileria</i> sp. (kudu), <i>T. buffeli</i>	Negative	Negative



F13	Farm 1, Pongola, KZN	F	+	<i>Theileria</i> sp. (kudu), <i>T. buffeli</i>	+	<i>Anaplasma marginale</i>
F14	Farm 1, Pongola, KZN	F	+	<i>Theileria</i> sp. (kudu), <i>T. buffeli</i>	+	<i>Ehrlichia</i> sp. Omatjenne
F15	Farm 1, Pongola, KZN	F	+	<i>Theileria</i> sp. (kudu), <i>T. buffeli</i>	+	<i>Ehrlichia</i> sp. Omatjenne, <i>Anaplasma bovis</i>
F16	Farm 1, Pongola, KZN	F	+	<i>Theileria</i> sp. (kudu), <i>T. buffeli</i>	Negative	Negative
F17	Farm 1, Pongola, KZN	F	+	<i>Theileria</i> sp. (kudu), <i>T. buffeli</i>	Negative	Negative
F18	Farm 1, Pongola, KZN	F	+	<i>Theileria</i> sp. (kudu), <i>T. buffeli</i>	+	Negative
F19	Farm 1, Pongola, KZN	F	+	Negative	+	Negative
F20	Farm 1, Pongola, KZN	F	+	Negative	+	Negative
F21	Farm 1, Pongola, KZN	F	+	Negative	+	Negative
F22	Farm 3, Pongola, KZN	F	+	<i>Theileria</i> sp. (kudu), <i>T. buffeli</i>	+	<i>Anaplasma marginale</i>
F23	Farm 3, Pongola, KZN	F	+	<i>Theileria</i> sp. (kudu), <i>T. buffeli</i>	+	<i>Ehrlichia</i> sp. Omatjenne
F24	Farm 3, Pongola, KZN	F	+	<i>Theileria</i> sp. (kudu), <i>T. buffeli</i>	+	<i>Ehrlichia</i> sp. Omatjenne
F25	Farm 3, Pongola, KZN	F	+	<i>Theileria</i> sp. (kudu), <i>T. buffeli</i>	+	<i>Ehrlichia</i> sp. Omatjenne
F26	Farm 3, Pongola, KZN	M	+	<i>Theileria</i> sp. (kudu), <i>T. buffeli</i>	+	<i>Ehrlichia</i> sp. Omatjenne, <i>Anaplasma marginale</i>
F27	Farm 3, Pongola, KZN	F	+	<i>Theileria</i> sp. (kudu), <i>T. buffeli</i>	+	<i>Ehrlichia</i> sp. Omatjenne
F28	Farm 3, Pongola, KZN	F	+	<i>Theileria</i> sp. (kudu), <i>T. buffeli</i>	+	<i>Ehrlichia</i> sp. Omatjenne



F29	Farm 3, Pongola, KZN	F	+	<i>Theileria</i> sp. (kudu), <i>T. buffeli</i>	+	<i>Ehrlichia</i> sp. Omatjenne, <i>Anaplasma</i> <i>marginale</i>
E8	Farm 4, Eastern Cape Province	M	+	<i>Theileria</i> sp. (kudu), <i>T. buffeli</i> , <i>T. bicornis</i> , <i>T. taurotragi</i>	Negative	Negative

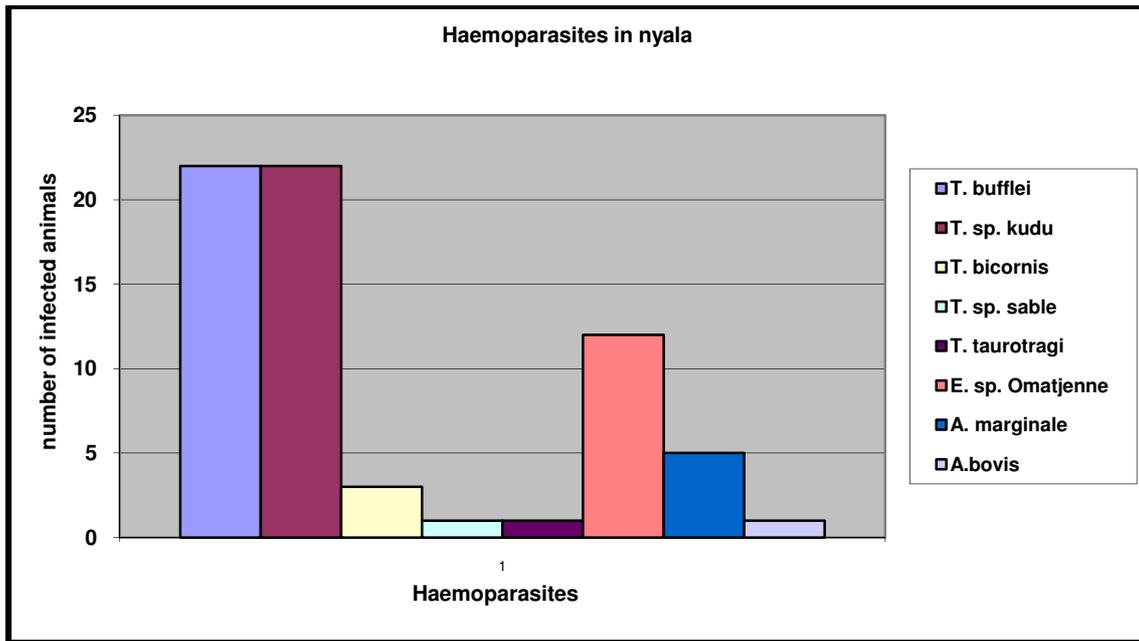


Figure 6: The various haemoparasites that were identified and number of animals infected with these haemoparasites.

5. DISCUSSION

Nyala that tested positive for haemoparasites were found on all farms where filter paper samples were taken. The infection was irrespective of gender, and adult as well as subadult animals were infected.

Of the 143 nyala specimens screened with the reverse line blot (RLB) hybridization assay, only samples on filter paper tested positive for the presence of haemoparasites while most EDTA blood samples (n = 113) – except for sample E8 from the Eastern Cape Province – tested negative. Twenty-seven of 30 samples (90%) on filter paper tested positive – most of them for several different haemoparasites.

5.1. HAEMOPARASITES PRESENT IN NYALA

Results gained from filter paper samples gave a good indication of which haemoparasites are carried by nyala. As animals were healthy at capture and translocated well, nyala seem to carry these haemoparasites subclinically and without ill effect.

Seeing the high number of positive animals from filter paper samples (90%), it would be fair to say that the prevalence of carriers of various tick-borne haemoparasites in nyala generally is high. This is not surprising, given the high tick burden that these animals are exposed to (Baker & Keep 1970; Horak *et al.* 1983; Horak *et al.* 1995).

Infections were mostly multiple, with various haemoparasites occurring in the same animal. The most common piroplasms found in nyala were *Theileria buffeli* and *Theileria* sp. (kudu), both of which were carried by 22 animals. It is also remarkable that these two infections always appeared together. Animals were found to carry *Theileria bicornis*, *Theileria* sp. (kudu) and *T. buffeli* in the Eastern Cape Province as well as in KZN and therefore it can be concluded that these piroplasms are very widely spread and common.

It remains to be established what role nyala play in the epidemiology of the various blood parasites and whether nyala are merely dead-end hosts or whether the parasites carried are numerous enough to infect vectors and therefore play a role in the epidemiology.

5.1.1. *Theileria buffeli*

Theileria buffeli, which is considered non-pathogenic, is transmitted by ticks of the genus *Haemaphysalis*, but other tick species are possibly involved in the transmission in Africa (Lawrence 2004). *Theileria buffeli* infection in cattle in Spain was demonstrated by means of the RLB hybridization assay (Garcia-Sanmartin *et al.* 2006). Thirty-eight percent of blood

samples contained *T. buffeli*, often as a mixed infection with other *Theileria* or *Babesia* species.

The RLB has also been used for detecting haemoparasites in cattle in Sicily (Georges *et al.* 2001). The results were similar to those of Garcia-Sanmartin *et al.* (2006). *Theileria buffeli* had a very high prevalence (up to 100%) and was often seen together with other infections. *Theileria buffeli* was also isolated from cattle in Turkey (Altay, Aydin, Uluisik, Aktas & Dumanli 2008), Michigan (USA) (Cossio-Bayugar, Pillars, Schlater & Holman 2002), Australia (Stewart, Standfast, Baldock, Reid & de Vos 1992) and Sudan (Salih, El-Hussein, Seitzer & Ahmed 2007). *Theileria buffeli* was further found in blood samples of 23 of 24 African buffalo tested in the Kruger National Park (Allsopp *et al.* 1999). Closely related *Theileria* species were found in small ruminants in China (Yin, Schnittger, Luo, Seitzer, & Ahmed 2007) as well as in sika deer (*Cervus nippon*) in Japan (Inokuma, Tsuji, SamJu, Fujimoto, Nagata, Hosoi, Arai, Ishihara & Okuda 2004).

Although this is the first report of *T. buffeli* in nyala, the fact that they are commonly carriers of *T. buffeli* is therefore not surprising. This is not of concern from a translocation or animal-disease point of view, however, as the organism is widespread and usually apathogenic or only causing mild disease (Lawrence 2004).

5.1.2. *Theileria* sp. (kudu)

Soon after translocation of greater kudu from the Eastern Cape Province to a game ranch near Mossel Bay several kudu died of a disease resembling theileriosis. A new *Theileria* [*Theileria* sp. (kudu)] could be identified by sequence analysis from one of these animals (Nijhof *et al.* 2005). *Theileria* sp. (kudu) is not known to be pathogenic to domestic animals and other wildlife. However, the fact that greater kudu presumably died of theileriosis after translocation indicates that *Theileria* sp. (kudu) can be the cause of disease in stressed animals. So far there have been no reports of nyala with clinical theileriosis. Nyala are known to suffer from translocation stress, however, and high, often unexplained, losses can occur after translocation. If captive or recently translocated nyala show typical signs of theileriosis such as anaemia, lymph node enlargement, petechiae, splenomegaly or lung oedema, clinical theileriosis due to *Theileria* sp. (kudu) should be considered as a differential diagnosis – especially since greater kudu and nyala are closely related, both belonging to the genus *Tragelaphus* (Skinner & Smithers, 1990).

5.1.3. *Theileria bicornis*

Theileria bicornis, originally described from healthy black rhinoceroses in South Africa, is not known to be pathogenic (Nijhof *et al.* 2003). In our study, *T. bicornis* was carried by three animals and occurred as mixed infections with other *Theileria* and *Ehrlichia* species. Although not much is known about *T. bicornis*, this finding shows that this *Theileria* species also has a broad host range and crosses the species barrier. The fact that *T. bicornis* was isolated from animals from KZN as well as from the Eastern Cape Province indicates a wide geographical spread.

5.1.4. *Theileria* sp. (sable)

Theileria sp. (sable) causes fatal clinical disease in roan and sable antelope in South Africa. Clinical signs were reported to consist of – amongst others – anaemia and icterus (Nijhof *et al.* 2005). *Theileria* sp. (sable) has also been isolated from healthy animals, such as African buffalo, African short-horn cattle in Tanzania, blesbok in Swaziland as well as from blue wildebeest, klipspringer and common reedbuck (Nijhof *et al.* 2005). It was also isolated from nyala and bushbuck in South Africa (Steyl *et al.* 2004). A *Theileria* species similar to *Theileria* sp. (sable) was isolated from red hartebeest in Namibia (Spitalska *et al.* 2005). The main vectors are possibly *R. evertsi evertsi* and *R. appendiculatus* (Steyl *et al.* 2004; Nijhof *et al.* 2005).

In our study, only one sample, originating from the Pongola area, tested positive for *Theileria* sp. (sable). As this organism can cause fatal disease in valuable antelope such as sable and roan, one has to be careful when introducing carrier animals onto a farm where sable and roan are bred and where *Theileria* sp. (sable) does not occur endemically.

At this stage, it is not clear if nyala play a role as carriers of *Theileria* sp. (sable) or if the finding was incidental. Nyala could merely be infected with this piroplasm as dead-end hosts without a role to play in the epidemiology and spread.

Nevertheless, until the role in the epidemiology has been established, roan and sable breeders should be careful with any new game introductions. *Theileria* sp. (sable) could be introduced with many different species, resulting in disease and losses in resident sable and roan antelope. Many game breeders have included nyala in their collections due to their aesthetic appeal and relatively high value. If sable and roan are kept in the vicinity, such a decision should be taken only after careful consideration. The endemic situation of *Theileria* sp. (sable) on the farm should be assessed. Measures to prevent spread of disease, such as dipping and vaccination of captive-bred animals, should be taken. Preventative treatment to eliminate the carrier status of animals that are to be introduced onto a not endemically infected farm should also be considered (Steyl *et al.* 2004).

5.1.5. *Theileria taurotragi*

Theileria taurotragi is a benign parasite of eland but readily transmissible to cattle. Severe disease consisting of lymph node enlargement, anaemia, a febrile reaction, respiratory distress and wasting of the animal occasionally develops in eland (Grootenhuis *et al.* 1980). The haemoparasite is transmitted by *R. appendiculatus*, *R. evertsi evertsi*, *R. pulchellus* and *R. zambeziensis* (Lawrence *et al.* 1994). Recovered cattle remain carriers for several months (Lawrence *et al.* 1994). Clinical signs in cattle usually consist of a mild febrile reaction. Occasionally nervous signs can develop (Lawrence *et al.* 1994).

Eland are considered to be the main wild reservoir host of *T. taurotragi* (Grootenhuis *et al.* 1980). In our study, one sample collected from a nyala in the Eastern Cape Province tested positive for *T. taurotragi*. Eland were not present on the farm where this animal was darted. This could indicate that species other than eland could serve as potential wildlife reservoirs of *T. taurotragi* for cattle. Whether the parasitaemia in the nyala was high enough and the carrier stage long enough to infect ticks is questionable, however, and would need further experimental investigation.

5.1.6. *Theileria parva*

As was to be expected, none of the nyala showed a positive reaction for *Theileria parva*. This is despite the fact that some of the samples were taken on farms where *Theileria parva*-infected buffalo were present.

Although reports exist that waterbuck (*Kobus defassa*) were found to be asymptomatic carriers of *T. parva* (Stagg *et al.* 1994), the only confirmed carrier of *Theileria parva* to date is the African buffalo, Asiatic buffalo (*Bubalus bubalis*) as well as cattle. If cattle are infected with *Theileria parva*, this usually results in diseases such as Corridor disease, East Coast fever and Zimbabwe theileriosis (Lawrence *et al.* 2005 a; Lawrence *et al.* 2005 b; Lawrence *et al.* 2005 c)

5.1.7. *Babesia* species

None of the animals tested was infected with any *Babesia* species, despite the fact that they are good hosts for *Boophilus* and *Rhipicephalus* ticks (Horak *et al.* 1995).

Babesia species described from wildlife include *B. bicornis* in black rhinoceros (Nijhof *et al.* 2003) and *B. irvinesmithi* as well as other new *Babesia* species in sable antelope (Martignalia 1930; Thomas *et al.* 1982, McInnes *et al.* 1991; Oosthuizen *et al.* 2008). *Babesia* was also found in a blood smear obtained from a dead bushbuck from Hluhluwe Game Reserve in

northern KZN (Bigalke *et al.* 1972). In the current study, however, none of the nyala carried any *Babesia* species.

On five occasions the PCR products failed to hybridize with any of the *Babesia* or *Theileria* species-specific probes, and only hybridized with the *Babesia* / *Theileria* genus-specific probe, suggesting the presence of a novel species or variant of a species. This warrants further investigation.

5.1.8. *Ehrlichia* sp. Omatjenne

Ehrlichia sp. Omatjenne, an *Ehrlichia*-like agent, was isolated from a *Hyalomma truncatum* tick from Omatjenne in the Otjiwarongo district of Namibia, an area free of *Amblyomma* ticks and therefore free of *E. ruminantium*, the cause of heartwater (Du Plessis 1990). After several passages of this agent through *Amblyomma* ticks, however, sheep developed severe signs of disease similar to heartwater.

In our study, the second-most common infection was with *Ehrlichia* sp. Omatjenne. Twelve animals carried this *Ehrlichia* sp., eleven as mixed infections with other haemoparasites. Northern KZN as well as regions of the Eastern Cape fall within the distribution range of *Hyalomma truncatum* (Walker, Bouattour, Camicas, Estrada-Peña, Horak, Latif, Pegram & Preston 2003), which could explain the occurrence of this rickettsia.

5.1.9. *Ehrlichia ruminantium*

Despite the fact that the sample areas in KZN as well as in the Eastern Cape were endemic regions for heartwater (Walker & Olwage 1987), none of the nyala tested seemed to be carriers of this rickettsia. A carrier status would be of concern when translocating nyala, as it could lead to the spread of heartwater into non-endemic areas such as North and South America, where potential vector ticks are present (Peter *et al.* 2002; Uilenberg 1982). As Peter *et al.* (2002) pointed out, the host range of *E. ruminantium* seems vast and infection with the agent has been proven in African buffalo, black wildebeest, blesbok, blue wildebeest, eland, giraffe, greater kudu, sable antelope, lechwe, sitatunga, springbok and steenbok, as well as in three non-African ruminants and two African rodents.

Artificially infected greater kudu showed no sign of disease and the infection did not establish in two out of five kudu (Peter *et al.* 1998). As kudu and nyala are relatively closely related – both belonging to the genus *Tragelaphus* (Skinner & Smithers 1990) – it is not too surprising, therefore, that none of the nyala examined carried *E. ruminantium*. This indicates that nyala naturally either do not develop a carrier stage at all or that the carrier stage is of short duration.

5.1.10. *Anaplasma marginale*

Anaplasma marginale is a widespread pathogen and gallsickness occurs endemically in most cattle-farming areas in southern Africa (Potgieter & Stoltz 2005), amongst them in north eastern KZN. Five animals, three of which were also carriers of *Ehrlichia* sp. Omatjenne carried *A. marginale*. All of the carriers of *A. marginale* were also infected with *Theileria* species. Most farms on which nyala were captured in the Pongola area had been used for cattle ranching not more than five to seven years previously or share a boundary with cattle-grazing areas. The vector of *A. marginale* is not completely clear, but the blue tick *Rhipicephalus (Boophilus) decoloratus* possibly is one of the main vectors of the disease, together with other tick species. However, mechanical transmission by biting flies and hypodermic needles is also possible (Potgieter & Stoltz 2005).

Anaplasma marginale has been found in game species on several occasions. It was implicated in the death of a giraffe (Augustyn & Bigalke 1974), but other game species did not seem to show clinical signs. A grey duiker infected with *A. marginale* developed an inapparent infection (Neitz & Du Toit 1932). One sable antelope showed *A. marginale* in a blood smear (Thomas *et al.* 1982). Blesbok, blue and black wildebeest were also found to be carriers of *A. marginale* (Kuttler 1984). In Kenya, Ngeranwa *et al.* (2008) discovered a high seroprevalence of *Anaplasma* species in game at the domestic livestock–wildlife interface. Species examined were eland, blue wildebeest, kongoni, impala, Thomson's gazelle, Grant's gazelle, giraffe and plains zebra. Prevalence varied from 75 to 100%. This indicates that game might play a significant role in the epidemiology of *Anaplasma* organisms and that wildlife could serve as a reservoir for infection of cattle.

This serves as the first report of *A. marginale* in nyala.

5.1.11. *Anaplasma bovis*

Anaplasma bovis was previously described as *Ehrlichia bovis* but reclassified by Dumler, Barbet, Bekker, Dasch, Palmer, Ray, Rikihisa & Rurangirwa (2001). According to Sumption & Scott (2005), who described it as *E. bovis*, *A. bovis* is the cause of bovine ehrlichiosis or a condition called Nofel in West Africa. *Anaplasma bovis* was isolated in 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, Rikihisa, Lin, Isogai, Tahara, Itagaki, Hiramitsu & Tajima 2006). Infection of cattle can lead to irregular fever, lymphadenopathy, depression and loss of condition. Fatally infected animals develop hydropericardium and central nervous signs. Inoculation of *E. bovis* into sheep has caused disease (Sumption & Scott 2005). The pathogen was diagnosed in South Africa in 1937 (Sumption & Scott 2005).

African tick vectors for *A. bovis* include *Hyalomma excavatum*, *Rhipicephalus appendiculatus* and *Amblyomma variegatum* (Sumption & Scott 2005). Serological cross-reaction with *E. ruminantium* has been reported (Dumler *et al.* 2001).

Only one nyala, originating from farm 1 in the Pongola area, tested positive for the presence of *A. bovis*. Also, in seven cases, PCR amplicons failed to hybridize with any of the *Ehrlichia* or *Anaplasma* species-specific probes, only hybridizing with the *Ehrlichia* / *Anaplasma* genus-specific probe present on the blot. Although these signals were very often weak and hardly visible, it still suggests the presence of a new species or variant of a species. This warrants further investigation.

5.2. THE DIFFERENCE IN RESULTS FROM EDTA BLOOD SAMPLES COMPARED TO BLOOD SAMPLES ON FILTER PAPER

The fact that mostly blood samples taken on filter paper turned out positive and nearly all blood samples taken as whole blood in EDTA turned out negative was an unexpected finding.

The DNA concentration extracted from filter paper samples is usually expected to be twenty times lower than the DNA concentration extracted from whole blood samples; therefore it was expected to rather find haemoparasites in the EDTA samples than in the filter paper samples (QIAamp ® DNA Mini Kit and QIAamp DNA Blood Mini Kit Handbook of 2003, QIAGEN, Southern Cross Biotechnologies). In order to make up for the predicted loss in DNA due to the use of blood spots, double the amount (5 µl) of DNA was added to the PCR reaction mixture of filter paper samples.

EDTA blood samples have to be stored chilled. The addition of anticoagulant such as EDTA is often not sufficient to prevent degradation of samples, especially in warmer climates (Duscher, Peschke, Wille-Piazzai & Joachim, 2009). Stability of DNA in whole blood is affected by changes of the storage temperature, even if the sample is always stored under chilled conditions (Tani, Tada, Sasai & Baba, 2007). The stability of DNA of *Babesia gibsoni* in whole blood with EDTA compared to blood spots on filter paper was evaluated by Tani *et al.* (2007). It was found that the stability of DNA in bloodspots stored at room temperature was superior to the stability when the sample was collected as whole blood and stored at -20°C.

Due to the field conditions in which the blood was collected and the initial storage of EDTA blood in a household freezer at -20°C for several months, it could be concluded that the DNA in whole blood samples deteriorated in such a way that eventually there was not enough parasite DNA left to be extracted.

The measurements of DNA by agarose gel electrophoresis and spectrophotometry after DNA extraction could have still been high due to the host DNA that is also measured in this case.

6. Conclusions

From this study one can draw the conclusion that nyala commonly carry multiple asymptomatic infections of various *Theileria* species, as well as of *Anaplasma* species and *Ehrlichia* species. This is not surprising, given their natural subtropical to tropical savannah bushveld habitat (Pfitzer & Kohrs 2005) and the multitude of ticks that these animals carry (Horak *et al.* 1995; Baker & Keep 1970). Haemoparasites identified from nyala in this study were: *Theileria buffeli*, *Theileria* sp. (kudu), *T. taurotragi*, *Theileria* sp. (sable), *T. bicornis*, *Ehrlichia* sp. Omatjenne, *Anaplasma marginale* and *A. bovis*. For most of these organisms, this was the first report of their occurrence in nyala. These results therefore also shed more light on the host range and distribution of the various haemoparasites.

The study shows that *T. bicornis*, recently described from black rhinoceroses (Nijhof *et al.* 2003), is by no means a parasite of rhinoceroses only. The same applies for *T. buffeli*, which had previously not been isolated from African antelopes but mainly from buffalo and domestic ruminants (Lawrence 2004). *Theileria taurotragi*, which was considered to be a haemoparasite of cattle and eland, was also found to occur in nyala (Lawrence *et al.* 1994).

Nyala can now also be listed as potential carriers of *A. marginale*, a haemoparasite that is widespread at the wildlife / livestock interface (Ngeranwa *et al.* 2008).

Several new questions have to be answered as a result of this study, such as what is the significance of nyala and other antelope species in the epidemiology of haemoparasites such as *T. taurotragi* and *A. marginale* – both of which are known to be pathogenic to cattle? Do nyala carry enough viable haemoparasites to infect vectors and play a role in the epidemiology of these various diseases or are these just incidental findings? This thesis confirms that nyala are carriers of *Theileria* sp. (sable). Their role in the epidemiology of this parasite and whether *Theileria* sp. (sable) could be introduced into sable-breeding facilities by introduction of nyala still has to be established and would be quite an important question – keeping in mind the numerous sable and roan-breeding projects that have been established and the high value and vulnerable conservation status of sable and roan antelope.

Theileria sp. (kudu) contributes to disease in stressed or immunocompromised kudu. Nyala are prone to translocation stress and it has to be established whether this piroplasm also plays a role in the death of translocated nyala.

Given the number of new haemoparasites that were discovered recently due to more sensitive and specific methods, the detection of new haemoparasites in some of these samples which only tested positive with the genus-specific RLB probes would not be

surprising (Lopez-Rebollar, Penzhorn, De Waal & Lewis 1999; Nijhof *et al.* 2003; Nijhof *et al.* 2005; Matjila *et al.* 2008; Oosthuizen *et al.* 2008; Oosthuizen, Allsopp, Troskie, Collins & Penzhorn 2009).

From these results, it also becomes clear that there seems to be a difference in results of samples taken on filter paper compared to EDTA blood samples. This is possibly due to the fact that despite most possible care to uphold an even chilling temperature, the field conditions, which were often complicated by numerous power failures, were not good enough and led to degradation of parasite DNA. As a consequence, recommending that blood samples for haemoparasite surveillance purposes should rather be taken on filter paper than as blood in EDTA tubes should be considered.

Finally, there are a multitude of known and unknown haemoparasites circulating in different game species, many of which seem to cross the species barrier. Most possibly the occurrence of haemoparasites also differs from area to area and is strongly influenced by the environment and the presence and abundance of vectors and hosts. The various haemoparasites from different areas and in different species could potentially be used to follow and confirm paths of migration or evolution of wild animals. They might even be of use for forensic purposes – to prove the origin of an individual animal.

When translocating wild and even domestic animals, measures should be taken to avoid the introduction of new piroplasms into areas where they have not occurred before. This is due to the fact that animals that are naïve to these piroplasms could potentially develop disease even if haemoparasites involved usually are apathogenic.

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