Occurrence of *Anaplasma* and *Ehrlichia* species in African buffalo (*Syncerus caffer)* in Kruger National Park and Hluhluwe-iMfolozi Park in South Africa

by

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Submitted in partial fulfillment of the requirements of the Master of Science degree in the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria

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DECLARATION

I declare that the dissertation, which I hereby submit for the Master of Science degree in the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria, to be my own work and has not been previously submitted by me for a degree at another tertiary institution.

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EM Debeila

November 2012

Dedication

Dedicated to my Parents Mr Samson Mosotho & Mrs Seipati Roseline Debeila

And to the memory of my late Grandparents
Mr Ishmael Lehlohonolo Makhaotse & Mrs Mmagomogase Ruth Debeila

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ABBREVIATIONS

A:	Adenine
ARC:	Agricultural Research Council
bp:	base pair
BLAST:	basic local alignment search tool
C:	Cytosine
°C:	degrees Celsius
DNA:	deoxyribonucleic acid
dNTP:	deoxynucleotide triphosphates
DVTD:	Department of Veterinary Tropical Diseases
EDTA:	Ethylenediaminetetraacetic acid
G:	Guanine
h:	hour
KNP:	Kruger National Park
µl:	microliter
mg:	milligram
ml:	millilitre
mM:	milli molar
min:	minute
ng:	nanogram
OVI:	Onderstepoort Veterinary Institute
pmol:	picomoles
PCR:	polymerase chain reaction
qPCR:	quantitative real time PCR
RLB:	reverse line blot
rRNA:	ribosomal ribonucleic acid
SDS:	sodium dodecyl sulphate
T:	Thymine
U:	enzyme units

Occurrence of *Anaplasma* and *Ehrlichia* species in African buffalo (*Syncerus caffer)* in Kruger National Park and Hluhluwe-iMfolozi Park in South Africa

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SUMMARY

Theileriosis, babesiosis, heartwater and anaplasmosis are considered to be amongst the most important tick-borne diseases of livestock in sub-Saharan Africa's tropical and subtropical regions resulting in extensive economic losses to farmers in endemic areas. It is well-known that the African buffalo (*Syncerus caffer*) is the natural reservoir host of various tick-borne haemoparasites of veterinary importance. In this study, the occurrence of tick-borne haemoprotozoan parasites (*Theileria*, *Babesia, Anaplasma* and *Ehrlichia* species) in buffalo from two geographically isolated national parks in South Africa (Kruger National Park and Hluhluwe-iMfolozi Park) was determined using the reverse line blot (RLB) hybridization assay. The RLB results revealed the presence of *Theileria*, *Babesia* and *Anaplasma* species either as single or as mixed infections. Although not detected with the RLB assay, 5% of the buffalo blood samples from the KNP tested positive for the presence of *Ehrlichia ruminantium* using the pCS20 real-time PCR assay. Previous studies on the occurrence of haemoparasites in the South African buffalo population have mainly focussed on the prevalence of *Theileria* species only. The finding on the presence of *Anaplasma, Ehrlichia* and *Babesia* species is therefore a novel contribution.

This study has confirmed the findings of previous studies that buffalo is the natural reservoir host of both pathogenic and non-pathogenic *Theileria* species namely, *T. parva*, *Theileria* sp. (buffalo), *T. mutans*, *T. velifera* and *T. buffeli*. In this study, the most frequently occurring

Theileria species detected in the KNP were *T. mutans* (81%), *Theileria* sp. (sable) (61%), *T. parva* (40%), *Theileria* sp. (buffalo) (13%) and *T. velifera* (11%). *Theileria buffeli* was not detected in the KNP. In the Hluhluwe-iMfolozi Park, the most occurring *Theileria* species were *T. mutans* (55%), *T. velifera* (54%), *T. parva* (53%), *Theileria* sp. (sable) (53%), *Theileria* sp. (buffalo) (49%) and *T. buffeli,* (49%). *Theileria* sp. (sable) causes fatal clinical disease in roan and sable antelope in South Africa and we can only speculate whether the presence of *Theileria* sp. (sable) DNA in the buffalo population was a true and/or incidental finding. An interesting finding was the presence of *Babesia occultans* DNA in 50% of the buffalo from the Hluhluwe-iMfolozi Park. *Babesia occultans* is the causative agent of a benign form of cattle babesiosis in South Africa and, to date; this organism has not been identified in wildlife in South Africa. The significance of this finding warrents further investigation and confirmation using gene cloning, sequencing and phylogenetic analysis.

Ehrlichia ruminantium has been reported to infect not only domesticated ruminants but also wild ruminants, however most wildlife species appear to carry the organism asymptomatically. In this study, we were not able to detect *E. ruminantium* DNA in any of the buffalo samples tested using the RLB hybridization assay. However, using the quantitative pCS20 real-time PCR assay we detected *E. ruminantium* DNA in 5% of the KNP samples. None of the Hluhluwe-iMfolozi Park samples tested positive for *E. ruminantium* using the real-time PCR assay. These results suggest that buffalo is not the natural reservoir host of *E. ruminantium*. However, a subclinical carrier state in buffalo has been experimentally shown to occur after tick transmission from carrier animals and further studies will have to be conducted to confirm whether this finding holds any potential risk to domestic animals.

In Southern Africa, two *Anaplasma* species are known to infect cattle, *A. marginale* and *A. centrale*. Clinical bovine anaplasmosis is usually caused by *A. marginale*; whilst *A. centrale* generally results in mild disease. Because there is partial cross immunity between the two species, *A. centrale* is used as a live vaccine for cattle in Israel, South Africa, South America and Australia. Apart from cattle, *Anaplasma marginale* has been described in wild ruminants which can become persistently infected serving as reservoirs for infection of susceptible hosts; it has been recovered from 10 wild ruminants. Subclinical occurrence of *A. marginale*, either natural or after artificial infection has been confirmed in the African buffalo and

various other wildlife species. In this study, the *Anaplasma* species detected from Hluhluwe-iMfolozi Park buffalo samples were *A. centrale* (75%), *A. marginale* (42%) and *Anaplasma (*formerly *Ehrlichia)* sp. Omatjenne (28%). DNA of these species was also detected in buffalo from KNP; *A. centrale* (49%), *A. marginale* (24%) and *Anaplasma (Ehrlichia)* sp. Omatjenne (5%). The presence of *A. marginale* in the buffalo population suggests that buffalo may be a factor in the epidemiology and spread of bovine anaplasmosis because, as reservoir hosts of *A. marginale*, they could serve as a source of infective blood for mechanical spread by various routes and biological transmission by ticks. Factors such as climate, host abundance, tick host diversity, and topography have, however, all been shown to also impact on the epidemiology of *A. marginale*.

Subsequently 64 samples were selected that either tested (i) positive for a specific *Anaplasma* spp. (*A. centrale, A. marginale* and/or *Anaplasma* (*Ehrlichia*) sp. Omatjenne) using the RLB assay, or (ii) in which the PCR products hybridized only with the *Anaplasma/Ehrlichia* genus-specific probes for molecular characterization by cloning and sequencing of the 16S rRNA gene. Aplification of the full-length and/or partial parasite 16S rRNA gene of any of the selected samples that previously tested positive for the presence of *Anaplasma* (*Ehrlichia*) sp. Omatjenne (using the RLB assay) or *E. ruminantium* (using the pCS20 real-time PCR assay) was unsuccessful. This was most probably due to low rickettsaemia. However, amplification of either the near full-length parasite 16S rRNA gene or a partial 16S rRNA gene from seven samples from the KNP and three from Hluhluwe-iMfolozi Park was successful. Results indicated that the obtained sequences of 12 of the 18 clones were highly similar to published *A. centrale* 16S rRNA gene sequences, four of the clones were highly similar to the published *A. marginale* sequences and the sequences of the remaining two clones were closely similar to *Anaplasma* (*Ehrlichia*) sp. strain Omatjenne. The observed sequence similarities were confirmed by phylogenetic analyses. An interesting finding was the presence of one full-length parasite 16S rDNA sequence that was 100% identical to that of the published *A. centrale* vaccine strain sequences. It is well known that *A. centrale* is widely used as live vaccine for the control of bovine anaplasmosis. The occurrence of *A. centrale* vaccine strain DNA in the South African buffalo population is therefore of great interest. It can only be speculated whether *A. centrale* has evolved in the African buffalo, and/or if buffalo act as natural reservoir hosts, or if is it merely being maintained in the

buffalo population by *in utero* transmission. This also serves as the first report of *Anaplasma (Ehrlichia)* sp. Omatjenne DNA in the African buffalo which warrents further investigation.

In conclusion, the findings suggest that buffalo is a natural reservoir of *Anaplasma* spp. infection and could play an important role in the epidemiology and spread of anaplasmosis and may represent a serious threat to the livestock industry.

CHAPTER 1

INTRODUCTION

The African buffalo (*Syncerus caffer*) is a member of the "Big Five" group of animals and is a sought-after commodity in South Africa. Buffalo populations in the national and provincial game parks in South Africa are estimated to have an intrinsic growth rate at around 12% per annum and they serve as a major tourist attraction. Smaller populations are also found in privately owned reserves or in buffalo breeding projects (Jolles, 2007). Buffalo are known to be a reservoir host for diseases such as foot and mouth disease (FMD), Corridor disease, bovine tuberculosis and brucellosis. The rising popularity of commercial game farms and private game reserves in South Africa has led to an increasing demand for certified "disease-free" buffalo. Buffalo movement is, however, strictly regulated by the South African veterinary authorities.

Theileriosis, babesiosis, heartwater and anaplasmosis are considered to be amongst the most important tick-borne diseases of livestock in sub-Saharan Africa, tropical and subtropical regions (Provost & Bezuidenhout, 1987; Rajput, *et al.,* 2005; Kocan, *et al.,* 2010) resulting in extensive economic losses to farmers in endemic areas (Uilenberg, 1981; Kocan, *et al.,* 2010). Theileriosis is a widespread disease of wild and domestic ruminants caused by tick-transmitted apicomplexan parasites of the genus *Theileria*. In sub-Saharan Africa, the most pathogenic and economically significant *Theileria* species is *T. parva*, which appears to have evolved in the African buffalo (syn. Cape buffalo) (*Syncerus caffer*) (Norval, *et al.,* 1992). Whilst causing only subclinical infection in buffalo, *T. parva* causes fatal disease in cattle. *Theileria parva* is the causative agent of East Coast fever (ECF), Corridor disease and January disease in cattle (Uilenberg, *et al.,* 1982; Perry, *et al.,* 1991; Norval, *et al.*, 1992). It is transmitted by three-host ixodid ticks *Rhipicephalus appendiculatus, R. zambeziensis* and *R. duttoni* (Lawrence, *et al*., 1994; Uilenberg, 1999). Corridor disease is a controlled disease in South Africa and veterinary authorities attempt to keep buffalo separated from livestock to prevent the spread of the disease (Stoltsz, 1989). Other reservoir hosts of *T. parva* are water buffalo (*Bubalus bubalis*) and waterbuck (*Kobus defassa*) (Stagg, *et al.,* 1994).

Bovine babesiosis is an economically important tick-borne disease of cattle in tropical and subtropical regions of the world (McCosker, 1981). The disease is primarily caused by two bovine intraerythrocytic protozoan parasites, *Babesia bovis* (Asiatic redwater) and *Babesia bigemina* (African redwater) which are mainly transmitted by the one-host ticks *Rhipicephalus decoloratus* and *R. microplus* (de Vos, *et al.,* 2004).

Bovine anaplasmosis is one of the most important diseases of ruminants worldwide causing significant economic losses in the livestock industries in the tropical and subtropical areas mainly due to the high morbidity and mortality in susceptible cattle herds (Kocan, *et al*., 2003). The primary causative agent is *Anaplasma marginale*, a Gram-negative obligate intracellular bacteria parasitizing erythrocytes (Rymaszewska & Grenda, 2008). It has been described in domestic and wild animals (cattle, water buffalo, bison, African antelopes, white-tailed deer and mule deer); clinical disease is most notable in cattle, but wild ruminants can become persistently infected serving as reservoirs for infection of susceptible hosts (Kocan, *et al*., 2003). It is an infectious but noncontagious disease which is spread through tick bites, mechanical transfer and/or transplacental transmission (Rymaszewska & Grenda, 2008).

Heartwater (or cowdriosis) is one of the most important tick-borne diseases of domestic ruminants (cattle, sheep and goats) in sub-Saharan Africa resulting in extensive economic losses to farmers in endemic areas (Provost & Bezuidenhout, 1987). The causative agent is *Ehrlichia ruminantium* (formerly known as *Cowdria ruminantium*), an intracellular rickettsial bacterium (Cowdry, 1925a; Dumler, *et al*., 2001). It is transmitted by three-host ticks belonging to the genus *Amblyomma* (Cowdry, 1925a; 1925b; 1926; Peter, *et al.,* 2002), primarily *Amblyomma variegatum* and *A. hebraeum* (Walker & Olwage, 1987). Mortality rates are high in susceptible cattle, sheep and goats and peracute, acute, subacute and clinically inapparent forms of heartwater have been reported (Van De Pypekamp & Prozesky, 1987).

Although *A. marginale* and *E. ruminantium* have been reported to infect wild ruminants and have been detected in wildlife, little is known about the susceptibility of wild ruminants to *A. marginale* and/or *E. ruminantium* infection or the role of wild ruminants in the epidemiology of the diseases caused. Deaths due to infection with *E. ruminantium* have been reported in

blue wildebeest (*Connochaetes taurinus*), springbok (*Antidorcas marsupialis*), bushbuck (*Tragelaphus scriptus*) (Oberem & Bezuidenhout, 1987), black wildebeest (*Connochaetes gnu*), blesbok (*Damaliscus dorcas phillipsi*) (Neitz, 1935), steenbok (*Raphicerus campestris*) (Jackson & Andrew, 1994), sitatunga (*Tragelaphus spekei*) (Okoh, *et al.*, 1986) and eland (*Taurotragus oryx*) (Young & Basson, 1973). *Anaplasma marginale* has been isolated from the East African blue and black wildebeest (Lohr & Meyer, 1973;). Serological evidence of anaplasmosis in buffalo has been observed (Kuttler, 1965) and a mild *A. marginale* isolate has been recovered from buffalo by inoculation of bovines (Potgieter, 1979). There are also two reports of acute *Anaplasma* infections in giraffe *(Giraffa camelopardalis)* in which severe clinical signs were seen (Lohr & Meyer, 1973; Augustyn & Bigalke, 1974). *Anaplasma marginale, A. centrale* and *A. ovis* have been recovered from blesbuck following experimental exposure to these organisms (Neitz, 1939). No mortalities due to anaplasmosis and/or heartwater have yet been documented in the African buffalo (Pfitzer, *et al.*, 2004). Conversely, in the case of *E. ruminantium* it was experimentally shown that a subclinical carrier state can occur in buffalo (Andrew & Norval, 1989; Wesonga, *et al.*, 2001). This would suggest that wild ruminants could play an important role in the epidemiology and spread of anaplasmosis and heartwater and that these animals could serve as reservoirs of infection which may represent a threat to the livestock industry (Andrew & Norval, 1989). Concern about transmission of infectious agents between wildlife and domestic livestock is increasing, especially in areas where free-ranging wildlife and cattle share common grazing grounds (Kocan, *et al.,* 2010).

As limited or no information is available on the *Anaplasma* and *Ehrlichia* spp. infection status of African buffalo, the primary objective of this study was, therefore, to determine the occurrence of *Anaplasma* and *Ehrlichia* species in African buffalo blood samples collected from two game parks in South Africa. The specific objectives were:

1. Screening of buffalo blood samples for the presence of *Anaplasma* and *Ehrlichia* species using the reverse line blot (RLB) hybridization assay. Samples were simultaneously screened for the presence of *Theileria* and *Babesia* spp.
2. Screening of buffalo blood samples for the presence of *E. ruminantium* using a quantitative real-time PCR assay targeting the pCS20 gene.

3. Phylogenetic analysis by amplification, cloning and sequencing of the 16S rRNA gene of some of the *Anaplasma* species detected in the first two objectives.

CHAPTER 2

LITERATURE REVIEW

Theileriosis, babesiosis, heartwater and anaplasmosis are considered to be the most important tick-borne diseases of livestock in sub-Saharan Africa, tropical and subtropical regions (Provost & Bezuidenhout, 1987; Rajput, *et al.,* 2005; Kocan, *et al.,* 2010) resulting in extensive economic losses for farmers in endemic areas (Uilenberg, 1981; Kocan, *et al.,* 2010). The African buffalo is known to be the reservoir host of numerous important tick-borne pathogens of which some are carried asymptomatically, but when transmitted to susceptible animals, are known to cause severe disease (Andrew & Norval, 1989; Allsopp, *et al*., 1999; Kocan, *et al.,* 2010). Buffalo are the reservoir host for *Theileria parva*, causing East Coast fever (ECF), Corridor disease and January disease (Young, *et al.*, 1977). A mild *A. marginale* isolate has been recovered from buffalo by inoculation of bovines (Potgieter, 1979) and it has been experimentally shown that a subclinical *E. ruminantium* carrier state can occur in buffalo (Andrew & Norval, 1989; Wesonga, *et al.*, 2001). This would suggest that buffalo could also play an important role in the epidemiology and spread of anaplasmosis and heartwater and that buffalo could serve as reservoirs of infection which may represent a threat to the livestock industry (Andrew & Norval, 1989).

1. BOVINE ANAPLASMOSIS

Among tick-borne diseases, bovine anaplasmosis is one of the most important diseases of ruminants worldwide causing significant economic losses in the livestock industries in the tropical and subtropical areas mainly due to the high morbidity and mortality in susceptible cattle herds (Kocan, *et al*., 2003). The primary causative agent is *Anaplasma marginale*, a Gram-negative obligate intracellular bacterium parasitizing erythrocytes (Rymaszewska & Grenda, 2008). It was first described by Theiler in South Africa in 1909, as “marginal points” in bovine erythrocytes. The scientific name is based on its staining characteristics and location within the host cell, with “*Anaplasma*” referring to the lack of stained cytoplasm and “*marginale*” denoting the peripheral location of the organism in the host erythrocyte. In 1911,

Theiler isolated the less virulent *A. centrale* that was most often located centrally within erythrocytes. It is transmitted biologically by ticks, or mechanically by insects and others (De La Fuente, *et al.*, 2004, 2005; Molad, *et al.*, 2006). In South Africa, it is transmitted mainly by five Ixodid ticks namely: *Rhipicephalus decoloratus*, *R. microplus*, *Hyalomma marginatum rufipes*, *Rhipicephalus evertsi evertsi* and *R. simus* (Dreyer, *et al.*, 1998).

1.1 Taxonomic classification

The genus *Anaplasma* belongs to the family Anaplasmataceae (order Rickettsiales), a family of obligate intracellular organisms that are found exclusively within membrane-bound vacuoles in the host cell cytoplasm (Kocan, *et al.*, 2004). All members of the family are obligate intracellular bacteria that share similar morphologic characteristics, and, when they are pathogenic, they can cause similar clinical manifestations. Many members of the family have adapted to existence within arthropods; some are transmitted by tick bites and cause human infections (Dumler, *et al.*, 2001). The genus *Anaplasma* has recently been expanded to include three species transferred from the genus *Ehrlichia*: *A. phagocytophilum* (formerly *Ehrlichia phagocytophilum, E. equi*, HGE agent), infecting numerous species of mammals including humans; the ruminant pathogens *A. marginale* (type species), *A. centrale* (formerly *A. marginale* subspecies *centrale*), *A. ovis,* and *A. bovis* (formerly *Ehrlichia bovis*), and the canine pathogen *A. platys* (formerly *Ehrlichia platys*) (Dumler, *et al.*, 2001; Kocan, *et al.*, 2004).

Clinical bovine anaplasmosis is usually caused by *A. marginale*. *Anaplasma marginale* does not cause disease in humans (Aubry & Geale, 2011). Cattle can also be infected with *A. centrale*, which generally results in mild disease. *Anaplasma centrale* is used as a live vaccine for cattle in Israel, South Africa, South America and Australia (De La Fuente, *et al.*, 2005a). *Anaplasma bovis* is detected mainly in cattle, but also observed in small mammals which probably act as reservoir of *A. bovis* (Goethert & Telford, 2003a). It causes monocytic anaplasmosis; the clinical symptoms are most visible in calves (Uilenberg, 1997; Santos & Carvalho, 2006). *Anaplasma bovis* has been detected in Brazil, North America, Africa and Japan (Goethert & Telford, 2003b; Kawahara, *et al.*, 2006; Santos & Carvalho, 2006). In addition, *Anaplasma ovis*, the agent of ovine anaplasmosis, may cause mild to severe disease in sheep, deer and goats but is not infectious for cattle (Aubry & Geale, 2011). Its presence

has been confirmed in most regions of the world both in farm and wild animals (Kuttler, 1984).

1.2 Geographical distribution

Anaplasma marginale occurs in most tropical and subtropical areas throughout the world, including North and South America, Africa, the Caribbean, Russia, European countries bordering the Mediterranean, and the Middle and Far East causing a major constraint to cattle production and resulting in huge economic losses to farmers in endemic areas (Kocan, *et al.,* 2003). This wide and increasing distribution likely resulted from increased transportation of cattle with subsequent mechanical or biological transmission from asymptomatic persistently infected animals to susceptible ones (Kocan, *et al*., 2010). *Anaplasma centrale* was first described from South Africa (Theiler, 1911) as *A. marginale* subspecies *centrale* and has since been imported by other countries, including Australia and some countries in South America, South-East Asia and the Middle East, for use as a vaccine against *A. marginale.*

Anaplasmosis is widespread in South Africa with more than 99% of the total cattle population at risk (De Waal, 2000). Most of the cattle farming areas in South Africa occur in the endemic and epidemic areas of anaplasmosis as indicated in Figure 2.1 (De Waal, 2000). Incidence of disease mortalities in official veterinary reports indicate that 18% of all reported cattle mortalities in South Africa are due to the tick-borne diseases anaplasmosis, heartwater, theileriosis and babesiosis, of which anaplasmosis is responsible for approximately 3% (De Waal, 2000).

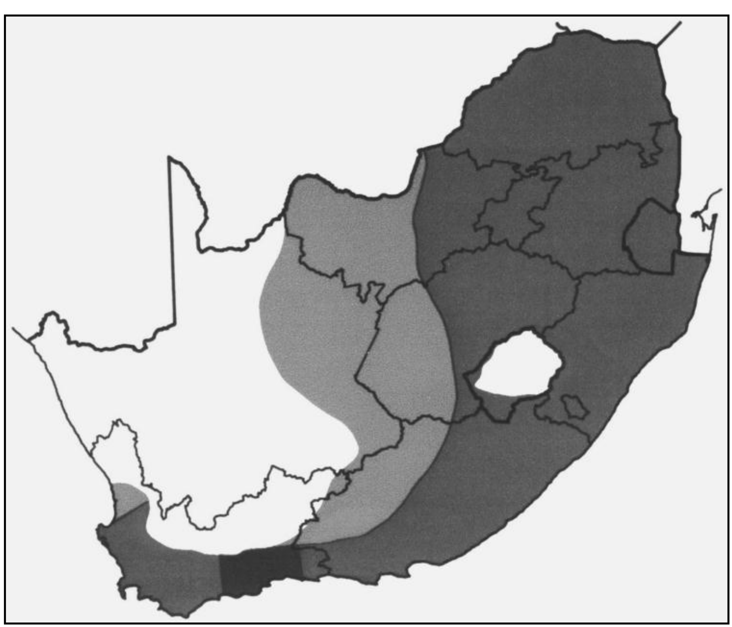

Figure 2.1: Endemic and epidemic areas of anaplasmosis in South Africa: endemic areas are indicated by the darker gray areas , epidemic areas are indicated by the lighter gray areas (De Waal, 2000).

1.3 Host occurrence

Anaplasma species infect only ruminants; cattle are naturally susceptible to *A. marginale* and *A. centrale* and sheep to *A. ovis* (Aubry & Geale, 2011). Little is known about the susceptibility of wild ruminants to infection by *A. marginale* and the role of wild ruminants in the epidemiology of bovine anaplasmosis. Wild ruminants can become persistently infected with *Anaplasma* species and can serve as reservoirs for infection of susceptible hosts (Kocan, *et al*., 2003). Except for two reports of acute anaplasmosis in giraffes, naturally occurring *A. marginale* infections among wild ruminants have not been clinically apparent (Augustyn & Bigalke, 1974; Davidson & Goff, 2001). The potential subclinical impacts of bovine anaplasmosis on wild ruminants, in terms of effects on survival or reproduction, are not known (Aubry & Geale, 2011).

Anaplasma marginale has been recovered from water buffalo (*Bubalus bubalis*), white-tailed deer (*Odocoileus virginianus*), mule deer (*Odocoileus hemionus hemionus*), black-tailed deer (*Odocoileus hemionus columbianus*), pronghorn (*Antilocapra americana americana*), Rocky Mountain elk (*Cervus elaphus nelsoni*), bighorn sheep (*Ovis canadensis canadensis*), black

wildebeest (*Connochaetes gnou*), blesbok (*Damaliscus dorcas phillipsi*), and duiker (*Sylvicapra grimmia grirnmi*) (Kuttler, 1984). Subclinical occurrence of *Anaplasma marginale*, either natural or after artificial infection, has been confirmed in the African buffalo (Potgieter, 1979), eland (Peirce, 1972; Ngeranwa, *et al*., 1998), black wildebeest (Neitz, 1935), blue wildebeest (Smith, *et al*., 1974), grey duiker (Neitz & Du Toit, 1932) and blesbok (Neitz & Du Toit, 1932). Blesbok are also susceptible to *A. centrale* infection (Neitz & Du Toit, 1932). The occurrence of unidentified *Anaplasma* spp., based on positive serological assays or presence of organisms visible on blood smear examination, has been reported in African buffalo (Brocklesby & Vidler, 1966), blue wildebeest (Brocklesby & Vidler, 1966; Kuttler, 1965; Löhr & Meyer, 1973; Burridge, 1975), Coke's hartebeest (*Alcelaphus buselaphus cokei*) (Löhr & Meyer, 1973), Thomson's gazelle (*Gazella thomsonii*) (Löhr & Meyer, 1973), Grant's gazelle (*Gazella granti*) (Löhr, *et al*., 1973), gerenuk (*Litocranius walleri*) (Brocklesby & Vidler, 1965), impala (*Aepyceros melampus*) (Kuttler, 1965; Löhr, *et al*. 1973), sable antelope (Grobler, 1981; Thomas, *et al*., 1982), waterbuck (Kuttler 1965; Löhr, *et al*. 1974) and giraffe (Brocklesby & Vidler, 1966; Löhr & Meyer, 1973; Augustyn & Bigalke, 1974; Löhr, *et al*. 1973). *Anaplasma ovis, A. centrale*, or other as yet unidentified *Anaplasma* species may well occur in other ruminants.. With the exception of black-tailed deer, the epidemiologic significance of anaplasmosis in wildlife has yet to be determined (Kuttler, 1984). The only wild animal in which *Anaplasma* is reported to produce serious clinical disease is the giraffe (Augustyn & Bigalke, 1974; Davidson & Goff, 2001).

1.4 Transmission

Anaplasma species are transmitted either mechanically or biologically by arthropod vectors. *Anaplasma marginale* is transmitted (i) biologically: infected erythrocytes are ingested by ticks; *A. marginale* replicates within the tick's gut and salivary glands and is subsequently transmitted via tick saliva into uninfected ruminants; (ii) mechanically: infected erythrocytes are transferred from infected to susceptible cattle by biting flies or blood-contaminated fomites, including needles or surgical instruments, without amplification of *A. marginale*; and (iii) transplacentally: infected erythrocytes move across the placenta in the uterus from infected cows to their offspring, without amplification of *A. marginale* (Aubry & Geale, 2011).

Reviews based on transmission experiments list up to 19 different ticks as capable of transmitting *A. marginale* experimentally (Kocan, *et al*., 2004; Marchette & Stiller, 1982). These are: *Argas persicus, Ornithodoros lahorensis, Boophilus calcaratus, Rhipicephalus annulatus, R. decoloratus, R. microplus, Dermacentor albipictus, D. andersoni, D. hunteri, D. occidentalis, D. variabilis, Hyalomma excavatum, H. rufipes, Ixodes ricinus, I. scapularis, Rhipicephalus bursa, R. evertsi, R. sanguineus* and *R. simus.* Note that experimental demonstration of vector competence does not necessarily imply a role in transmission in the field. However, *Rhipicephalus* species are clearly important vectors of anaplasmosis in countries such as Australia and countries in Africa, and some species of *Dermacentor* are efficient vectors in the USA (OIE *Terrestrial Manual* 2008).

Five tick species are capable of transmitting *A. marginale* in South Africa namely *Rhipicephalus decoloratus, R. microplus, R. evertsi evertsi, Hyalomma marginatum rufipes* and *Rhipicephalus simus* (Potgieter, *et al.,* 1981). The main biological vectors of *A. centrale* appear to be multihost ticks peculiar to Africa, including *R. simus.* The common cattle tick *Rhipicephalus microplus* has not been shown to be a vector. This is of relevance where *A. centrale* is used as a vaccine in *Rhipicephalus microplus*-infested regions.

Various other biting arthropods have been implicated as mechanical vectors. Mechanical transmission of *A. marginale* by bloodsucking diptera of the genera *Tabanus*, *Stomoxys*, and mosquitoes (Ewing, 1981; Potgieter, *et al.,* 1981) have been reported. This form of mechanical transmission is considered to be the major route of dissemination of *A. marginale* in areas of Central and South America and Africa where tick vectors do not occur (Ewing, 1981) and where *Rhipicephalus microplus*, the tropical cattle tick, does not appear to be a biological vector of *A. marginale* (Figueroa, *et al.,* 1998). Mechanical transmission of *A. marginale* also occurs when infected blood is transferred to susceptible cattle via blood-contaminated fomites or mouthparts of biting flies (Ewing, 1981). *Anaplasma marginale* also can be transmitted during vaccination against other diseases unless a fresh or sterilised needle is used for injecting each animal. Similar transmission by means of unsterilised surgical instruments has been described (OIE *Terrestrial Manual* 2008).

Transplacental transmission of *A. marginale* may contribute to the epidemiology of bovine anaplasmosis in some regions as this form of transmission occurs when infected erythrocytes

move across the placenta in the uterus from infected cows to their offspring (Kocan, 1986). In a study conducted in South Africa, a 15.6% incidence of *in utero* transmission of *A. centrale* or *A. marginale* infections was reported among 77 calves born to splenectomized and intact cows, chronically infected or undergoing primary reactions during the first, second or third trimester of gestation (Potgieter & Van Rensburg, 1987). Transplacental transmission has also been reported in 32 out of 37 calves born to cows which had been affected with clinical anaplasmosis, confirmed in the laboratory, within the last 2 months of gestation. All the cows recovered following treatment for 5 days with tetracycline (Salabarria & Pino, 1988). Transplacental transmission of *A. marginale* may therefore contribute to the epidemiology of this disease in some regions (Kocan, *et al.,* 2003).

1.5 Life cycle

The developmental cycle of *A. marginale* is complex and coordinated with the tick feeding cycle (Figure 2.2) (Kocan, 1986; Kocan, *et al.,* 1992a,b). Infected erythrocytes ingested by ticks with the blood-meal provide the source of *A. marginale* infection for tick gut cells after which many other tick tissues become infected, including the salivary glands from where the rickettsiae are transmitted to vertebrates during feeding (Kocan, 1986; Kocan, *et al.,* 1992a, b). Two forms of *A. marginale*, reticulated and dense forms, are found in infected tick cells. Reticulated forms appear first and are the vegetative stage that divides by binary fission. The reticulated form changes into the dense form, which is the infective form and can survive extracellularly (Kocan, *et al.,* 2003). Cattle become infected with *A. marginale* when the dense reticulated form is transmitted during tick feeding via the salivary glands (Kocan, *et al.,* 2003). The disease is characterized by fever, severe anaemia, jaundice, brownish urine, loss of appetite, dullness or depression, rapid deterioration of physical condition, muscular tremors, constipation, yellowing of mucous membranes and laboured breathing (Bram, 1975; Kocan, *et al.,* 2010).

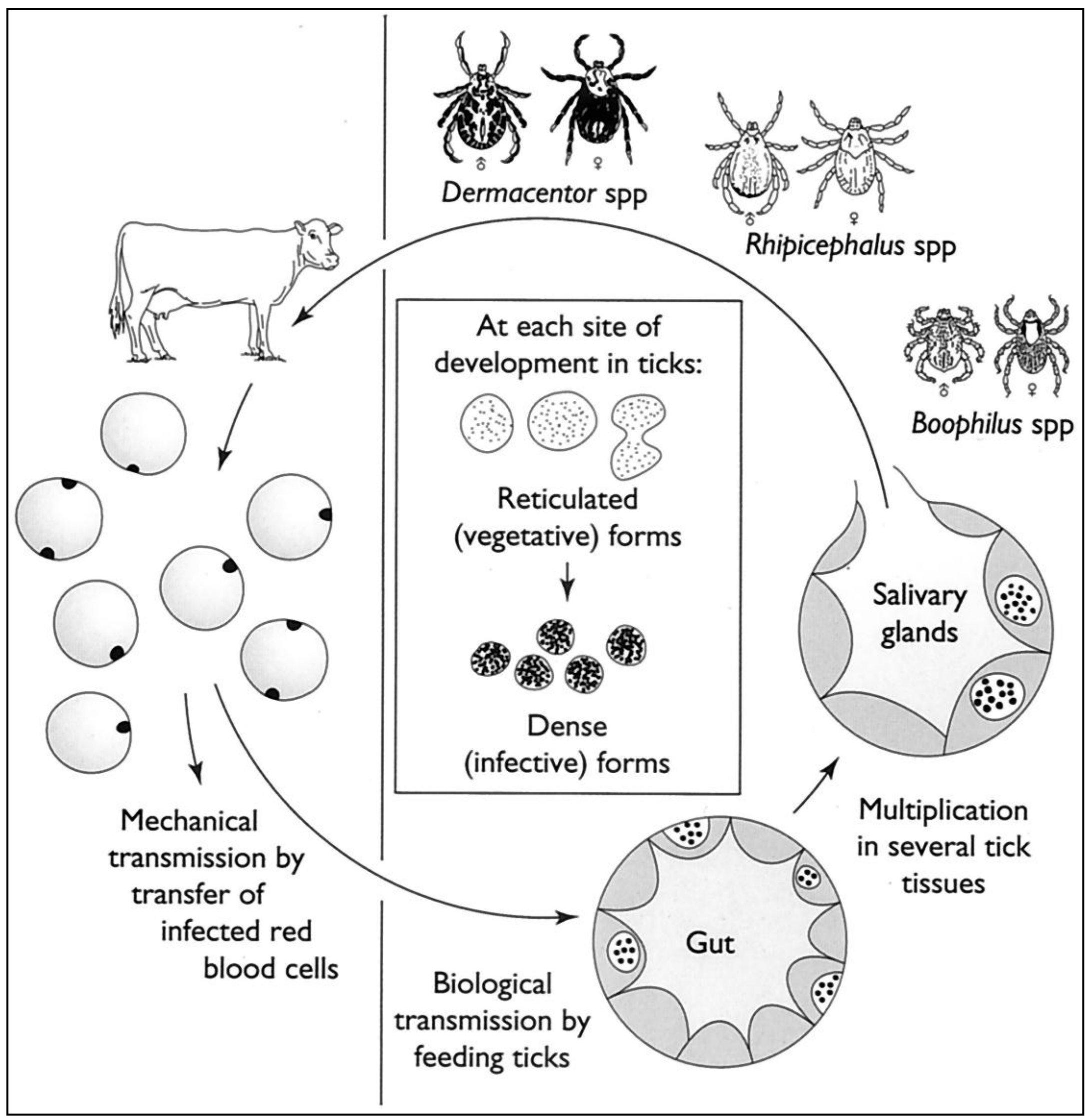

Figure 2.2: Schematic representation of the development cycle of *A. marginale* in cattle and ticks. (Taken from Kocan, *et al.*, 2004)

1.6 Control strategies

Control methods for anaplasmosis include (i) arthropod control, (ii) chemoprophylaxsis, (iii) vaccination (preimmunization with live vaccines and immunization with inactivated vaccines), (iv) prevention of iatrogenic transmission and (v) maintenance of *Anaplasma*-free herds through import and movement control, testing, and elimination of carrier cattle. Control measures implemented vary with geographic location, and depend on availability, cost, and the feasibility of application. Vaccination has been an effective means of preventing

outbreaks of anaplasmosis, but these vaccines (live and inactivated), are dependent on bovine blood as the source of infection or antigen (Kocan, *et al*., 2000).

In South Africa, anaplasmosis is controlled either by tick control (where acaricides are used to prevent tick transmission of the disease) or by vaccination (using a "blood vaccine" with a method known as infection and treatment) (Van der Merwe, 1987; Uilenberg, 1990). Apart from vector control being labour-intensive and expensive, environmental pollution is also becoming an important issue, and repeated application of acaricides can result in the development of resistant tick populations (Aubry & Geale, 2011). It also bears the risk of creating a susceptible population of cattle in endemic areas (Aubry & Geale, 2011). Tick control by acaricides is widely practiced in Africa (Kocan, *et al*., 2000).

Antibiotic therapy includes the use of tetracycline drugs (tetracycline hydrochloride, chlortetracycline, oxytetracycline, and doxycycline), imidocarb, and gloxazone. Chemotherapy is directed toward prevention of clinical anaplasmosis and does not prevent cattle from becoming persistently infected. Also, cattle receiving antibiotic therapy may not be cleared of infection (Kocan, *et al.*, 2000). Antimicrobial therapy is mostly used on healthy animals as control of active infections to limit the clinical effects of infection and treatment of clinical anaplasmosis (Potgieter & Stoltsz, 2004). Oxytetracycline is the most commonly used drug in South Africa, although imidocarb is also used for the treatment of acute anaplasmosis infections (Potgieter & Stoltsz, 2004; Aubry & Geale, 2011). The primary administration over prolonged periods of tetracycline, oxytetracycline, minocycline, and chlortetracycline, as well as the oral administration of chlortetracycline can sterilize *Anaplasma* infections (De Waal, 2000). This form of control does not reliably eliminate persistent infections, and there is no evidence that it prevents cattle from becoming infected with *A. marginale* (Kocan, *et al.,* 2000).

Development of long-term immunity by vaccination has been used extensively for control of anaplasmosis throughout most of the world and it represents the most effective control measure for anaplasmosis (reviewed by Palmer *et al*. in 1989). *Anaplasma centrale*, isolated by Sir Arnold Theiler in the early 1900s (Theiler, 1911), is currently the most widely used live vaccine strain for control of bovine anaplasmosis and it is used in several parts of the world, including Africa, Australia, Israel and Latin America (Aubry & Geale, 2011; Kocan,

et al., 2000). Vaccines for control of anaplasmosis include two major types (i) live and (ii) inactivated. Both have relied on *A. marginale* from infected bovine erythrocytes as the antigen source and both vaccine types induce protective immunity that mutes or prevents clinical disease, but neither types prevents cattle from becoming persistently infected with *A. marginale* (Aubry & Geale, 2011). *Anaplasma centrale* live vaccines were used to protect cattle imported from England to South Africa in early 1900s and have since been in use in tropical and subtropical regions (Potgieter, 1979; Losos, 1986; Pipano, *et al*., 1985; Turton, *et al*., 1998). Live vaccines involve exposure of cattle via inoculation with erythrocytes infected with *A. centrale* or with attenuated *A. marginale*. Another strategy has been to infect calves with *A. marginale* and then treat them with tetracycline at the onset of elevated body temperature or the detection of parasitemia (Potgieter, 1979; Aubry & Geale, 2011). This method requires close monitoring of cattle and may not be applicable for use in large herds as control of post-inoculation reactions is often unsuccessful in preventing acute disease (Kuttler & Todorovic, 1973; Kocan, *et al.,* 2010). Inactivated vaccines have several advantages over live vaccines; for example, they have low risk of contamination with undesirable infectious agents, can be inexpensively stored and generally cause minimal post-inoculation reactions. Disadvantages of inactivated vaccines include the need for yearly boosters, high cost of purification of *A. marginale* from erythrocytes and the lack of cross-protection among isolates from widely separated geographic areas (Kocan, *et al.,* 2010). In addition, the protective immunity afforded by inactivated vaccines is usually less than that of live vaccines (Kocan, *et al.,* 2010*).*

In the absence of a national eradication policy, as is the case for many countries, maintenance of *Anaplasma*-free herds is possible at the herd level, especially in areas where the prevalence of infection is low (Aubry & Geale, 2011). In areas where anaplasmosis is not endemic, anaplasmosis control has been effected by maintenance of *A. marginale*-free herds to avoid importation of anaplasmosis carriers that could serve as a source of infection (Kocan, *et al*., 2000). This control method is however dependent on the availability of specific and very sensitive diagnostic techniques that are able to reliably detect low level infection in carrier cattle.

1.7 Diagnosis

Diagnosis of bovine anaplasmosis is generally conducted with the examination of Giemsa-stained blood smears from clinically infected animals, during the acute phase of the disease (Decaro, *et al.,* 2008). Identification of these parasites by microscopy is based on the position of inclusion bodies within the erythrocyte, with *A. marginale* and *A. centrale* inclusions marginally and centrally located, respectively (Decaro, *et al.,* 2008). Examination of blood smears is, however, not reliable for detecting pre-symptomatic or carrier animals. In these instances, the infection is generally diagnosed by serologic demonstration of antibodies with confirmation by molecular detection methods.

Several serological tests have been employed extensively for epidemiological studies: complement fixation (CF) test, capillary agglutination assay, card agglutination test (CAT), indirect fluorescent antibody (IFA) test, as well as various enzyme-linked immunosorbent assays (ELISA) such as a cELISA, indirect ELISA and dot ELISA. The two serological tests currently preferred for identifying infected animals are the cELISA and the CAT (OIE, 2008; Kocan, *et al.*, 1992a, b; De La Fuente*, et al.,* 2005a, b). The most accurate serological test currently available for identifying *Anaplasma*-infected cattle is a cELISA that uses a monoclonal antibody (MAb) specific for MSP5 as this assay specifically detects the presence of serum antibodies that target a surface protein, MSP5 of *Anaplasma* spp. (Knowles, *et al.,* 1996; Torioni, *et al.,* 1998; Strik, *et al.,* 2007). However, the test cannot differentiate between *A. marginale* and some of the other *Anaplasma* species, because they all express the MSP5 antigen (Visser, *et al.,* 1992) and induce antibodies recognized by the MSP5-specific MAb.

Nucleic-acid-based tests have also been developed that are capable of detecting the presence of low-level infection in carrier cattle and tick vectors. These include conventional polymerase chain reaction (PCR), PCR-based hybridization assays and real-time PCR assays. PCR assays targeted at the *Anaplasma* msp4 and/or msp1a genes have been used to differentiate isolates of *A. marginale*, which is useful to track the origin of an outbreak, and to differentiate between different species of *Anaplasma* such as *A. marginale* and *A. centrale* (De La Fuente, *et al*., 2001; Lew, *et al*., 2002).

A reverse line blot (RLB) hybridization assay that combined both PCR and hybridization, has been developed to simultaneously detect and differentiate *Theileria, Babesia, Anaplasma* and

Ehrlichia species in infected animals (Gubbels, *et al*., 1999; Bekker, *et al*., 2002). PCR products are hybridized on a blot on which species-specific probes are covalently linked and the products are visualized with chemiluminescence after thoroughly washing of the membrane. This assay has since been used extensively in epidemiological surveys of tick-borne haemoparasites (Georges, *et al*., 2001; Almeria, *et al*., 2001; Oura, *et al*.. 2004; Nijhof, *et al*., 2003; Salih, *et al*., 2007; Matjila, *et al*., 2008; Oosthuizen, *et al*., 2008, 2009; Bhoora, *et al*., 2010; Bosman, *et al*., 2007; Chaisi, *et al*., 2011).

Recently, quantitative real-time PCR (qPCR) was successfully applied to the detection and quantification of *A. marginale* DNA (msp1b gene) in the blood of naturally infected cattle (Carelli, *et al*., 2007). The test was proven to be highly specific as there were no cross-reactions with other haemoparasites of ruminants (*A. centrale, A. bovis, A. phagocytophilum, B. bigemina, Theileria buffeli*). Subsequently, the *A. marginale* qPCR was modified by adding a primer/probe set specific for *A. centrale*, thus obtaining a duplex assay for simultaneous detection of both *Anaplasma* spp. in the same reaction (Decaro, *et al*., 2008).

The gold standard for the demonstration of *A. marginale*-free blood is the sub-inoculation of blood from the suspect animal into a splenectomized calf that is highly susceptible to infection (Coetzee, *et al*., 2006). This method is, however, costly and raises welfare issues, as the splenectomized calves become very ill after sub-inoculation of infected blood and often have to be euthanized (OIE, 2008).

2. HEARTWATER

Heartwater (cowdriosis) is an acute, fatal, non-contagious, infectious, tick-borne rickettsial disease of ruminants (Provost & Bezuidenhout, 1987). The causative agent is *Ehrlichia ruminantium* (formerly known as *Cowdria ruminantium*), an intracellular rickettsial bacterium (Cowdry, 1925a; Dumler, *et al*., 2001). It is transmitted by three-host ticks belonging to the genus *Amblyomma* (Cowdry, 1925a, b; 1926; Peter, *et al.,* 2002), primarily *A. variegatum* and *A. hebraeum* (Walker & Olwage, 1987). Mortality rates are high in susceptible cattle, sheep and goats and peracute, acute, subacute and clinically in apparent forms of heartwater have been reported (Van De Pypekamp & Prozesky, 1987; OIE, 2007). In South Africa, *E. ruminantium* is transmitted by *A. hebraeum,* and occurs mainly in the

Limpopo, Mpumalanga, KwaZulu-Natal and in the Eastern Cape provinces of South Africa (Walker & Olwage, 1987).

2.1 Taxonomic classification

The genus *Ehrlichia* belongs to the family Anaplasmataceae, in the order Rickettsiales (Dumler, *et al.*, 2001). *Ehrlichia* species are small (0.5 to 1.5 µm), Gram-negative, pleomorphic, obligate intracellular bacteria that reside and replicate in membrane-bound vacuoles of eukaryotic cells (Rikihisa, 1991). They are known to infect and cause disease in both humans and various animal species. *Ehrlichia* organisms are vector-borne; they replicate in the tick vector and are horizontally transmitted from infected cells in vectors to the blood cells of animals or humans.

The genus *Ehrlichia* includes five species. Three species (*E. canis* (type species), *E. ewingii*, and *E. chaffeensis*) cause infection in dogs and humans; *E. muris* is a murine pathogen, and *E. ruminantium* (formerly *Cowdria ruminantium*) causes heartwater disease in domestic ruminants (Dumler, *et al.*, 2001).

2.2 Geographic distribution

Heartwater occurs in nearly all the sub-Saharan countries of Africa where *Amblyomma* ticks are present and in the surrounding islands: Madagascar, Reunion, Mauritius, Zanzibar, the Comoros Islands and Sao Tomé. The disease is also reported in the Caribbean (Guadeloupe, Marie-Galante and Antigua) (Perreau, *et al.*, 1980), from where it threatens the American mainland (OIE, 2008). The map in Figure 2.3 shows the areas at risk from heartwater in sub-Saharan Africa, with the approximate numbers of domestic ruminants in those areas. The total is approximately 150,000,000 animals at risk, of which 114,000,000 (76%) are in the red areas of greatest potential exposure to tick challenge.

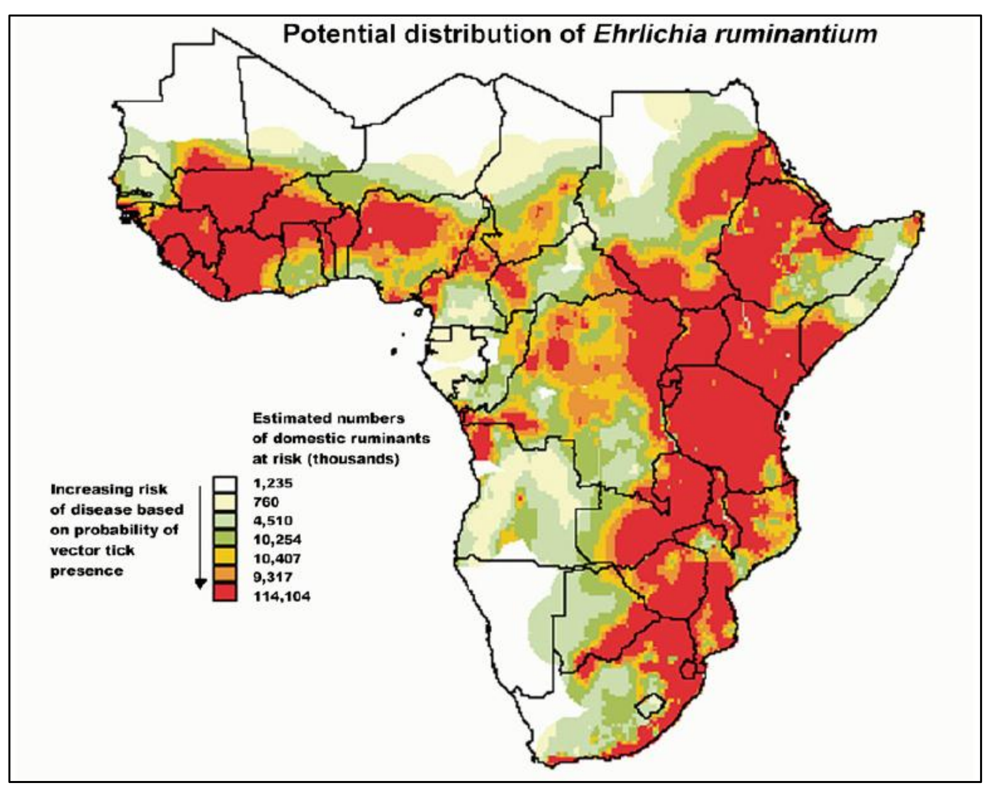

Figure 2.3: Potential distribution of *Ehrlichia ruminantium* in sub-Saharan Africa, based on habitat suitability for the vector tick species. (Taken from Allsopp, 2010 who took it with permission from a poster by Minjauw, *et al*., DFID-Animal Health Programme, CTVM, University of Edinburgh, Roslin, Midlothian, EH25 9RG, Scotland.)

The disease was first recognized in South Africa in the 19th century, where its tick-borne nature was determined in 1900, but the organism itself was not demonstrated until 1925, when it was recognized to be a rickettsia, initially named *Rickettsia ruminantium*. It was thus the first species of what are now known as *Ehrlichia* to be discovered, and most of the early work to elucidate the nature of the organisms, and its reservoirs and vectors, was performed in South Africa (Allsopp, 2010). In South Africa the *Amblyomma hebraeum* tick that exclusively transmits the disease is present in the Eastern Cape (former Transkei), KwaZulu-Natal, Mpumalanga, Limpopo, North West Province and Gauteng (north of the Magaliesberg). The tick is absent from the highveld and semi-desert areas of South Africa because it is too cold (and probably too dry) for the tick to survive (Howell, *et al.,* 1987; Walker, *et al*., 2003 Norval & Horak, 2004).

2.3 Host occurrence

It is thought that the first historical record of heartwater was made in 1838 by the South African pioneer Louis Trichardt, who described a fatal disease of sheep in South Africa (Neitz, 1947; Camus & Barré, 1988; Provost & Bezuidenhout, 1987). Until 1902, heartwater was believed to be a specific disease of sheep and goats (Lounsbury, 1902) after which it became apparent that cattle were also susceptible. However, mortality in sheep and goats is three times higher than in cattle (Yunker, 1996). Water buffalo (*Bubalis bulbalis*) are also susceptible and can die from heartwater (Mammerickx, 1961).

Ehrlichia ruminantium has been reported to infect not only domesticated ruminants but also wild ruminants (Pfitzer, *et al.*, 2004), but most wildlife species appear to carry the organism asymptomatically (Peter, *et al.* 2002). Deaths due to infection with *E. ruminantium* have been reported in blue wildebeest, springbok, bushbuck (Oberem & Bezuidenhout, 1987), black wildebeest, blesbok (Neitz, 1935), steenbok (Jackson & Andrew, 1994), sitatunga (Okoh, *et al.*, 1986) and eland (Young & Basson, 1973). No mortalities due to heartwater have yet been documented in the African buffalo (Pfitzer, *et al.*, 2004). However, it was experimentally shown that a subclinical carrier state can occur in buffalo (Andrew & Norval, 1989; Wesonga, *et al.*, 2001), as well as in eland, kudu, blue wildebeest, giraffe (Peter, *et al.*, 1998), sable antelope and blesbuck (Peter, *et al.*, 1999). This would suggest that wild ruminants could play an important role in the epidemiology and spread of heartwater and that these animals could serve as reservoirs of infection which may represent a threat to the livestock industry (Andrew & Norval, 1989). Although the infection status of this agent in wildlife might complicate heartwater control efforts, it may also serve to maintain a state of endemic stability (Yunker, 1996).

2.4 Transmission

Heartwater is transmitted by ticks of the genus *Amblyomma*. At least twelve *Amblyomma* spp. can transmit *E. ruminantium*. The tropical bont tick, *Amblyomma variegatum*, is the major vector in Africa and the Caribbean. Other known vectors include *A. hebraeum* (southern Africa), *A. lepidum* (East Africa and Sudan), *A. astrion* and *A. pomposum*. *Amblyomma sparsum, A. gemma, A. cohaerans, A. marmoreum* and *A. tholloni* are capable of transmitting experimental infections. *Amblyomma maculatum* and *A. cajennense*, both American tick

species, can transmit *E. ruminantium* in the laboratory but have not been implicated in natural infections.

In sub-Saharan Africa, *E. ruminantium* is transmitted by the "bont" ticks *A. hebraeum* and *A variegatum;* adult ticks preferably infest cattle but can also be attracted to sheep, goats and horses. Adults and nymphae usually attach on the belly or udder, while the nymphae and larvae can be located on the head and ears. *Amblyomma hebraeum* and *A. variegatum* are three-host ticks: both larvae and nymphae need to engorge on a separate host before dropping off to moult. All three life cycle stages (larvae, nymphae and adult ticks) can be infected with *E. ruminantium*, but only the nymphae and adult ticks are capable of transmitting heartwater from one ruminant to another (Cowdry, 1925a; 1925b; Daubney, 1930; Walker, 1962; 1984; 1990).

2.5 Life cycle

The life cycle of *E. ruminantium* include the reproduction stages taking place in both ixodid ticks, acting as vectors, and vertebrate animals. After an infected tick bite, the initial replication of the organism takes place in the reticuloendothelial cells in lymph nodes of the host. The organism enters into the blood and adheres to arterial endothelial cells (Du Plessis, 1970). After contact of *E. ruminantium* with endothelial cell membranes, the organism is taken up by phagocytosis whereafter replication takes place. The parasite multiplies by binary fission and by endosporulation inside the membrane-bound vacuole in the cytoplasm of endothelial cells. *Ehrlichia ruminantium* colonies establish in the host cells within 24 hours and logarithmic growth occurs before the elementary bodies (EBs) are released into the blood stream after rupturing the endothelial cells (Marcelino, *et al.,* 2005). Once in the blood stream the EBs can infect new endothelial cells. This cycle repeats itself until the animal starts to develop a fever (febrile reaction). Ticks are infected with EBs during the release phase, while feeding on the animal (Prozesky, 1987).

2.6 Control strategies

In South Africa, heartwater is controlled either by tick control where acaricides are used to prevent tick transmission of the disease or by vaccination using a "blood vaccine" with a method known as infection and treatment (Van der Merwe, 1987; Uilenberg, 1990). In the case of vaccination, the blood of donor animals infected with *E. ruminantium* is used to infect

susceptible livestock. Once a temperature response is detected these animals are treated with tetracycline. Although this vaccination procedure is fairly successful, it is laborious as each animal's temperature must be closely monitored every day after the infection was initiated. Also, the isolate used (Ball3) does not completely cross-protect against all the various field strains of *E. ruminantium* (Jongejan, *et al.,* 1988; Collins, *et al.,* 2003). Distribution of the vaccine in rural areas is also a problem as delivery relies on an uninterrupted cold chain.

The use of attenuated, inactivated and recombinant *E. ruminantium* vaccines has also been investigated. Although attenuated vaccines have shown promise, they must still be tested using natural tick challenge with virulent *E. ruminantium* genotypes in the field (Jongejan, 1991; Jongejan, *et al.,* 1991; Zweygarth, *et al.,* 2005). Inactivated vaccines have shown limited protection in sheep, goats and cattle and do not seem to be a commercially viable option (Martinez, *et al*., 1994; Mahan, *et al*., 1995; Martinez, *et al*., 1996; Totté, *et al*., 1997; Mahan, *et al.,* 1998; Mahan, *et al.,* 2001; Bell-Sakyi, *et al.,* 2002). Numerous attempts have been made to develop a recombinant DNA vaccine and although some protection was observed in mice, protective results were not reproducible (Nyika, *et al.,* 1998). A recombinant DNA vaccine known as 1H12 cocktail and which consists of four *E. ruminantium* (Welgevonden) open reading frames (ORFs) provides 100% protection against a virulent *E. ruminantium* (Welgevonden) needle challenge in sheep (Pretorius, *et al*., 2007). However, this vaccine was not protective when used in the field against a natural infection tick challenge as only 20% protection was obtained (Pretorius, *et al*., 2008).

2.7 Diagnosis

Microscopic examination of stained brain smears (Prozesky, 1987) is still the most reliable method used to confirm heartwater at *post mortem*. *Ehrlichia ruminantium* occurs as clumps of coccoid to pleomorphic organisms inside capillary endothelial cells. They are often found close to the nucleus, and may be in a ring or horseshoe formation. Serological diagnostic methods used include the immuno-fluorescence assay (IFA) and enzyme-linked immunosorbent assay (ELISA); however, these are not completely reliable as immunological cross-reactions with homologous antigens from other *Ehrlichia* species do occur (Du Plessis, *et al.,* 1993; Savadye, *et al.,* 1998). DNA-based PCR and probe tests are most often used to confirm heartwater infection in animals, the targets being the pCS20 genomic region and the 16S ribosomal RNA (rRNA) gene of the organism (Waghela, *et al.,* 1991; Allsopp, *et al.,*

1997; 1999). A reverse line blot (RLB) hybridisation assay, based on the 16S rRNA gene, is also in use (Bekker, *et al.,* 2002) and able to detect and simultaneously distinguish between *Ehrlichia* species. Quantitative real-time PCR was also recently developed for the detection of *E. ruminatium* using the pCS20 gene. In this assay, the pCS20 quantitative real-time PCR Taq Man probe is used (Doyle, *et al.,* 2005; Steyn, *et al.,* 2008).

3. CORRIDOR DISEASE

Theileriosis is a widespread disease of wild and domestic ruminants caused by tick-transmitted apicomplexan parasites of the genus *Theileria* (Uilenberg, 1999). In sub-Saharan Africa, the most pathogenic and economically significant *Theileria* species is *T. parva*, which appears to have evolved in the African buffalo (syn. Cape buffalo) (*Syncerus caffer*) (Norval, *et al.,* 1992). Whilst causing only subclinical infection in buffalo, *T. parva* causes fatal disease in cattle. *Theileria parva* is the causative agent of East Coast fever (ECF) (in eastern and central Africa), Corridor disease (in South Africa) and January disease (in Zimbabwe) in cattle (Young, *et al.*, 1977; Uilenberg, *et al.,* 1982, Uilenberg, 1999; Perry, *et al.,* 1991). It is transmitted by the three-host ixodid ticks *Rhipicephalus appendiculatus, R. zambeziensis* and *R. duttoni* (Lawrence, *et al*., 1994; Uilenberg, 1999). In addition to *T. parva*, the African buffalo is the natural host of the relatively benign *Theileria mutans* and the apathogenic *Theileria velifera*, both of which are transmitted by *Amblyomma hebraeum* (Norval, *et al.,* 1992). Corridor disease which derives its name because it was first identified in the 'corridor' between the Hluhluwe and iMfolozi game reserves in KwaZulu-Natal province of South Africa, is a controlled disease in South Africa and veterinary authorities try to keep buffalo separated from livestock to prevent the spread of the disease (Uilenberg, 1999; Lawrence, *et al.,* 1994).

In South Africa, *T. parva* is endemic in buffalo in the Kruger National Park (KNP) and the Hluhluwe-iMfolozi Park as well as on neighboring game parks (Collins, 1997; Mashishi, 2002). Because Corridor disease is a controlled disease in South Africa, it is required that buffalo undergo a series of parasitological, serological and molecular diagnostic tests before they are certified "*T. parva*-free" and fit for translocation. This includes microscopic examination of blood smears, the indirect fluorescent antibody test (IFAT) and a *T. parva* specific real-time PCR assay (Sibeko, *et al*., 2008).

4. BOVINE BABESIOSIS

Bovine babesiosis is a tick-borne disease of cattle that is caused by more than one intraerythrocytic protozoa of the genus *Babesia*, order Piroplasmida, phylum Apicomplexa (McCosker, 1981). The clinical manifestation of this disease is characterized by anemia, fever, hemoglobinuria, and marked splenomegaly and sometimes death. This leads to economic losses especially in the milk and meat production industries, which in turn impacts on the international cattle trade (Bock, *et al.,* 2004; OIE, 2008; Terkawia, *et al.,* 2011). Cattle that recover from acutes infection, both naturally or after chemotherapy, remain persistently infected and serve as a reservoir for transmission (Bock, *et al.,* 2004) and risk for future outbreaks in the absence of proper disease management strategies (Terkawia, *et al.,* 2011).

Babesia bovis and *B. bigemina* are two of the species affecting cattle and they are the most economically important species worldwide (Bock, *et al*., 2004; OIE, 2008). These two species are transmitted by the one-host ticks of the genus *Rhipicephalus* (Potgieter, 1977). *Rhipicephalus decoloratus* transmits only *Babesia bigemina,* while *R. microplus* transmits both *B. bigemina* and *B. bovis* (Potgieter, 1977). *Babesia bovis* causes more severe disease in susceptible cattle when the disease is associated with nervous symptoms because of the sequestration of infected erythrocytes in cerebral capillaries, resulting in low parasitemia in the circulating blood; while *B. bigemina* causes milder disease, resulting primarily in anemia with higer levels of parasitemia (Ristic, 1981; Bock, *et al.* 2004; Terkawia, *et al.,* 2011).

CHAPTER 3

MATERIALS AND METHODS

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1. Sample collection and DNA extraction

One hundred blood samples spotted on filter paper and 100 whole blood samples (in EDTA vacutainers) previously collected from buffalo in the Kruger National Park (KNP) and the Hluhluwe-iMfolozi Park (KwaZulu-Natal province), South Africa, were available for this study. Genomic DNA was extracted from the filter paper blood spots using the QIAmp DNA extraction kit (Qiagen, Hilden, Germany) and from whole blood using the High Pure Template Preparation kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturers' instructions. DNA was eluted in 100 µl elution buffer and stored at -20°C.

2. PCR amplification and RLB hybridization

The *Anaplasma* and *Ehrlichia* genus-specific forward primer Ehr-F (5'-GGA ATT CAG AGT TGG ATC MTG GYT CAG-3') (Schouls, *et al.,* 1999) and biotin-labelled reverse primer Ehr-R (5'-Biotin-CGG GAT CCC GAG TTT GCC GGG ACT TYT TCT-3') (Bekker, *et al.*, 2002) were used to amplify a 492 to 498 bp fragment of the parasite V1 hypervariable region of the 16S rRNA gene. For the simultaneous detection of *Theileria* and *Babesia* spp., the V4 hypervariable area of the parasite 18S 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') (Nijhof, *et al.*, 2005). The PCR reaction mixture consisted of 12.5 µl of Platinum® Quantitative PCR SuperMix-UDG (which contained of 3 mM $MgCl_2$, 200 µM each of dGTP, dATP and dCTP, 400 µM dUTP, 0.75 U Platinum® Taq DNA polymerase and 0.5 U uracil deoxy-glycosylase) (Invitrogen, The Scientific Group, South Africa), 0.1 µM of each primer and 2.5 µl of DNA to a total volume of 25 µl. For DNA extracted from blood spotted on filter paper, 5 µl of DNA was used. DNA from a *T. parva* positive buffalo (KNP102) as described by Sibeko *et al*. (2008) and DNA from a dog which tested positive for *Ehrlichia canis* infection (RLB 524/07) were used as positive controls. Water was used as

negative control. A touchdown PCR thermocycler program for *Ehrlichia/Anaplasma* and *Babesia/Theileria*, as described in by Nijhof *et al.* (2003) (Table 3.1), was used after which 5 μl of the PCR product was examined on a 2% agarose gel that was stained with ethidium-bromide and visualized on an ultra-violet transilluminator.

Table 3.1: Thermocycler program for *Ehrlichia/Anaplasma* and *Babesia/Theileria* touchdown RLB-PCR.

No of cycles	Duration	Temperature in °C	
1 cycle	3 min	37°C	Activation of UDG
1 cycle	10 min	94°C	Inactivation of UDG & activation of *Taq*
2 cycles	20 min 30 sec 30 sec	94°C 67°C 72°C	Denaturation Annealing Elongation
2 cycles	20 sec 30 sec 30 sec	94°C 65°C 72°C	Denaturation Annealing Elongation
2 cycles	20 sec 30 sec 30 sec	94°C 61°C 72°C	Denaturation Annealing Elongation
2 cycles	20 sec 30 sec 30 sec	94°C 61°C 72°C	Denaturation Annealing Elongation
2 cycles	20 sec 30 sec 30 sec	94°C 59°C 72°C	Denaturation Annealing Elongation
40 cycles	20 sec 30 sec 30 sec	94°C 57°C 72°C	Denaturation Annealing Elongation
2 cycles	7 min	72°C	Final extension

3. Reverse line blot (RLB) hybridization assay

The PCR products were analysed using the RLB hybridization technique as described by Nijhof *et al.* (2005).

3.1 Preparation of membrane

An in-house membrane was prepared containing the relevant genus- and species-specific probes as listed in Table 3.2. In preparation of this membrane a Biodyne C blotting membrane was activated (Pall Biosupport, Ann Arbor, Mich.) by incubating it for 10 min in 10 ml freshly prepared 16% (wt/vol) 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide (EDAC) (Sigma, St Louis, Mo.) at room temperature. After washing the membrane for 2 min with demineralized water, it was placed in a MN45 miniblotter (Immunetics, Cambridge, Massachusetts). The genus- and species-specific probes (0.25 pmol/µl) (containing a N-terminal N-(trifluoracetamidehexyl-cyanoethyl,N,N-diisopropyl phosphoramidite [TFA]) – C6 amino linker) were diluted to a final concentration of 2 pmol /150 µl (0.13 pmol/µl) and covalently linked to the membrane by filling the miniblotter slots with the oligonucleotide dilutions, then incubated at room temperature for 2–3 min. After the incubation, the oligonucleotide solutions were aspirated. The membrane was then inactivated by incubation in 100 ml of a 100 mM NaOH solution for 8 minutes at room temperature then finally washed in 100 ml 2X SSPE/0.1% sodium dodecyl sulfate (SDS) for 5 min at 60°C.

Table 3.2: List of genus- and species-specific probes used during the RLB hybridization assay. (Symbols indicate degenerate positions: R = A/G, W = A/T, K = G/T)

Oligonucleotide probe	**Sequence (5' – 3')**	**Reference**
Ehrlichia/Anaplasma genus-specific	GGG GGA AAG ATT TAT CGC TA	Bekker, *et al.*, 2002
Anaplasma bovis	GTA GCT TGC TAT GAG AAC A	Bekker, *et al.*, 2002
Anaplasma centrale	TCG AAC GGA CCA TAC GC	RLB manual, Isogen
Anaplasma (Ehrlichia) sp. *Omatjenne*	CGG ATT TTT ATC ATA GCT TGC	Bekker, *et al.*, 2002
Anaplasma marginale	GAC CGT ATA CGC A GC TTG	Bekker, *et al.*, 2002
Anaplasma phagocytophilum	TTG CTA TA A AGA AT A A TT AG T G G	Bekker, *et al.*, 2002
Ehrlichia canis	TCT GGC TAT AGG AAA TTG TTA	Bekker, *et al.*, 2002,
Ehrlichia chaffeensis	ACC TTT TGG TTA TAA ATA ATT GTT	RLB manual, Isogen
Ehrlichia ruminantium	AGT ATC TGT TAG TGG CAG	RLB manual, Isogen
Theileria/Babesia genus-specific	TAA TGG TTA ATA GGA RCR GTT G	Gubbels, *et al.*, 1999
Theileria genus-specific1	ATT AGA GTG TTT CAA GCA GAC	Nijhof[a] (unpublished)
Babesia genus-specific 1	ATT AGA GTG TTT CAA ACA GGC	Nijhof (unpublished)
Babesia genus-specific 2	ACT AGA GTG TTT CAA ACA GGC	Nijhof (unpublished)
Babesia bicornis	TTG GTA AAT CGC CTT GGT C	Nijhof, *et al.*, 2003
Babesia bigemina	CGT TTT TTC CCT TTT GTT GG	Gubbels, *et al.*, 1999
Babesia bovis	CAG GTT TCG CCT GTA TAA TTG AG	Gubbels, *et al.*, 1999
Babesia caballi	GTG TTT ATC GCA GAC TTT TGT	Butler, *et al.*, 2008

Babesia canis	TGC GTT GAC GGT TTG AC	Matjila, *et al.,* 2004
Babesia vogeli	AGC GTG TTC GAG TTT GCC	Matjila, *et al.,* 2004
Babesia divergens	ACT RAT GTC GAG ATT GCA C	Nijhof, *et al,* 2003
Babesia felis	TTA TGC TTT TCCGAC TGG C	Bosman, *et al.,* 2007
Babesia gibsoni	CAT CCC TCT GGT TAA TTT G	Nijhof, *et al.,* 2003
Babesia major	TCC GAC TTT GGT TGG TGT	Georges, *et al.,* 2001
Babesia microti	GRC TTG GCA TCW TCT GGA	Nijhof, *et al.,* 2003
Babesia occultans	CCT CTT TTG GCC CAT CTC GTC	He, *et al.,* 2012
Babesia sp. (sable)	GCT GCA TTG CCT TTT CTC C	Oosthuizen, *et al.,* 2008
Babesia sp. Xinjiang	GCG GGT TTC GTC TAC TTC GCT TTG T	He, *et al.,* 2012
Theileria annulata	CCT CTG GGG TCT GTG CA	Georges, *et al.,* 2001
Theileria bicornis	GCG TTG TGG CTT TTT TCT G	Nijhof, *et al.,* 2003
Theileria buffeli	GGC TTATTT CGG WTT GAT TTT	Gubbels, *et al.,* 1999
Theileria equi	TTC GTT GAC TGC GYT TGG	Butler, *et al.,* 2008
Theileria lestoquardi	CTT GTG TCC CTC CGG G	Schnittger, *et al.,* 2004
Theileria mutans	CTT GCG TCT CCG AAT GTT	Gubbels, *et al.,* 1999
Theileria parva	GGA CGG AGT TCG CTT TG	Nijhof, *et al.,* 2003
Theileria separata	GGT CGT GGT TTT CCT CGT	Schnittger, *et al.,* 2004
Theileria sp. (buffalo)	CAG ACG GAG TTT ACT TTG T	Oura, *et al.,* 2004
Theileria sp. (kudu)	CTG CAT TGT TTC TTT CCT TTG	Nijhof, *et al.,* 2005
Theileria sp. (sable)	GCT GCA TTG CCT TTT CTC C	Nijhof, *et al.,* 2005
Theileria taurotragi	TCT TGG CAC GTG GCT TTT	Gubbels, *et al.,* 1999
Theileria velifera	CCT ATT CTC CTT TAC GAG T	Gubbels, *et al.,* 1999

3.2 RLB hybridization

Prior to using the membrane, it was first incubated for 5 min in 10 ml 2 X SSPE/0.1% SDS at room temperature. A volume of 25 µl of PCR product was diluted to an end volume of 150 µl 2 X SSPE/0.1% SDS. The products were denatured for 10 min at 100°C in a thermocycler, cooled immediately on ice and centrifuged. The membrane was placed in a miniblotter, with slots perpendicular to the line pattern of applied probes. Slots were filled with diluted PCR product and the empty slots were filled with 2 X SSPE/0.1% SDS, to avoid cross flow. The miniblotter was incubated for 60 min at 42°C for hybridization to take place. Samples were removed by aspiration, thereafter the membrane was removed from the blotter. The membrane was washed twice in preheated 2 X SSPE/0.5% SDS for 10 min at 50°C in a water bath with gentle shaking. It was then incubated with 10 ml 2 X SSPE/0.5% SDS + 2.5 µl streptavidin-POD (peroxidase-labeled) conjugate (Roche Diagnostics, South Africa) (1.25 U) for 30 min at 42°C. The membrane was then washed twice again in preheated 2 X

SSPE/0.5% SDS for 10 min at 42°C in a water bath with gentle shaking. The membrane was washed again twice with 2 X SSPE for 5 min at room temperature under gentle shaking. The membrane was then incubated for 1 min in 10 ml of ECL detection fluid (DNA Thunder™, Perkin Elmer, Separation Scientific, South Africa). The membrane was covered with overhead sheet and air bubbles were removed by rolling a tube over the sheet. It was placed between 2 (clean) overhead sheets and placed in the exposure cassette. The X-ray film (X-OMAT™ Blue XB-1, Kodak, Separation Scientific, South Africa) was exposed for 5-20 min and then 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.

3.3 Stripping of the membrane:

After use, all PCR products were stripped from the membrane by washing the membrane twice in 1% SDS for 30 min at 100°C. After washing, the membrane was rinsed in 20 mM EDTA (pH 8.0) for 15 min at room temperature under gentle shaking. The membrane was then stored in fresh EDTA solution at 4°C in a sealed container for further use.

4. Amplification, purification and cloning of the full-length 16S rRNA gene

The primers fD1 (5'- AGA GTT TGA TCC TGG CTC AG -3') and rP2 (5'- ACG GCT ACC TTG TTA CGA CTT -3') (Weisburg, *et al.,* 1991) were used for the amplification of the near full length 16S rRNA gene of *Anaplasma* and *Ehrlichia* species found. The High Fidelity PCR Master System (Roche Diagnostics, Mannheim, Germany) was used to prepare PCRs according to the manufacturer's instructions. The reaction mixture consisted of 2.5 µl template DNA (~75 ng), 1.5 mM $MgCl_2$, 200 µM dNTPs, High Fidelity Enzyme blend (concentration unknown), 0.1 µM of each primer and nuclease-free water to a total volume of 25 µl. Amplification consisted of an initial denaturing at 94°C for 2 min followed by 40 cycles of 94°C for 30 sec, 60°C for 45 sec and 72°C for 1 min, with a final extension at 72°C for 7 min. PCR amplicons were purified to remove any impurities that might interfere with further analysis by using the QIAquick Purification kit (Qiagen) following the manufacturer's instructions. DNA was eluted in 20 µl elution buffer (EB). The purity and concentration of the DNA were determined spectrophotometrically. Purified PCR amplicons were then cloned into the pGEM® - T Easy Vector (Promega, Madison, WI, USA) and transformed into competent *E. coli* JM109 cells. The recombinant plasmids were isolated using the High Pure

Plasmid Isolation Kit (Roche Diagnostics, Mannheim, Germany) following the manufacturer's instructions. Plasmid DNA was eluted in 30 µl elution buffer and the concentration was determined spectrophotometrically. The plasmid DNA was then stored at -20°C prior to sequencing.

5. Nested PCR, amplification and cloning of the V1 region of the 16S RNA gene of detected *Ehrlichia* and *Anaplasma* species

The primers Ehr16SD (5'- GGT ACC YAC AGA AGA AGT CC –'3) and EBR3 (5-TTG TAG TCG CCA TTG TAG CAC-'3) were used for the first PCR and EbR2 (5'-TGC TGA CTT GAC ATC ATC CC-'3) and Ehr16SD where used for the second nested PCR (Brown, *et al.,* 2001) to amplify a 726 bp fragment of the 16S RNA gene. Amplification by PCR was performed in a 25 µl reaction mixture containing 1 µl of YellowSub (GENEO BioProductions, Hamburg, Germany), 5 µl template DNA, 200 µM dNTPs, 20 pmol of each primer, and 0.625 U of Taq DNA polymerase (Bio-Rad Laboratories) in a 5 X reaction buffer containing 1.65 mM $MgCl_2$. The nested PCR reaction mixture contained 0.5 µl of the primary PCR product. A standard PCR cycling programme was used: 40 and 25 cycles for the first and second round of PCR, respectively with an initial cycle of 92°C for 30 sec, 62°C for 45 sec, 72°C for 60 sec and final extension at 72°C for 3 min. PCR amplicons were purified to remove any impurities that might interfere with further analysis by using the QIAquick Purification kit (Qiagen) following the manufacturer's instructions. DNA was eluted in 20 µl elution buffer. PCR amplicons were then cloned into the TA pCR®2.1-TOPO vector (Invitrogen, USA) according to the manufacturer's manual. Five µl of the PCR product was examined on a 2% agarose gel that was stained with ethidium-bromide and visualized using an ultra-violet transilluminator. Recombinant plasmids were extracted and stored as described above.

6. Sequencing and phylogenetic analysis

Sequencing was performed using the ABI BigDye™ Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems), 350 ng plasmid DNA and 3.2 pmol of primer. The near full-length 16S rRNA gene sequences were sequenced using the universal primers fD1 and rP2. The vector primers SP6 (5'-TTA TAC GAC TCA CTA TAG GG-3') and T7 (5'-TAT TTA GGT GAC ACT ATA-3') were also used. An ABI3100 genetic analyzer located at the Agricultural Research Council within the Onderstepoort Veterinary Institute (ARC-OVI)

(South Africa) sequencing facility was used to analyze the purified products. For the partial sequences of the 16S RNA gene, primers Ehr16SD and EbR2 were used. The Flanders Institute for Biotechnology (VIB) sequencing department of the University of Antwerp, Belgium was used for sequencing using an ABI PRISM® Genetic Analyser (Perkin Elmer ABI, CA, USA).

The obtained sequences were assembled and edited using the GAP4 program of the Staden package (version 1.6.0 for Windows) (Bonfield, *et al.,* 1995; Staden, *et al.,* 2000). Searches of databases for homologous sequences were performed using BLASTn (Altschul, *et al.,* 1990). A multiple sequence alignment, which included 16S rRNA gene sequences of related genera available in GenBank, was performed using ClustalX (version 1.81 for Windows) (Thompson, *et al.,* 1997). The alignment was truncated to the size of the smallest sequence using BioEdit v7 (Hall, 1999). The TrN + I +G model was determined to be the best-fit for the data by using the Modeltest v3.7 software package (Posada & Crandall, 1998) and was subsequently used in the construction of the phylogenetic trees. Phylogenetic trees were constructed by the neighbor-joining (Saitou & Nei, 1987), maximum parsimony and maximum likelihood methods using PAUP* v4b10 (Swofford, 2003). Distance and parsimony methods were done in combination with the bootstrap method (Felsenstein, 1985) using 1 000 replicates/tree for each method. Bayesian analysis was done using MrBayes v3.1.2 (Ronquist & Huelsenbeck, 2003). In all instances, the 16S rRNA sequences of *Rickettsia rickettsii* (accession nr: U11021) was included as an outgroup to root the phylogenetic trees. All consensus trees were edited using the MEGA 4 (Tamura, *et al.,* 2007) software package. The GenBank accession numbers of the 16S rRNA gene sequences used to construct the phylogenetic tree are presented in Table 3.3.

Table 3.3: The 16S rRNA gene sequences used in the phylogenetic analyses.

Accession number	Taxonomic classification	Designation	Location	Source
EF520687	*A. centrale*	Strain 1	Italy	Bovine
EF520689	*A. centrale*	Strain 14	Italy	Bovine
EF520688	*A. centrale*	Strain 8	Italy	Bovine
EF520690	*A. centrale*	Strain 16	Italy	Bovine
EF520686	*A. centrale*	Strain CC	Italy	Bovine
AF309869	*A. centrale*	Strain Israel	South Africa	Bovine
AF414869	*A. centrale*	NA*	South Africa	*Rhipicephalus simus* tick
AF414868	*A. centrale*	Strain vaccine	Australia	Vaccine strain from South Africa
AF318944	*A. centrale*	Strain vaccine	South Africa	Bovine
DQ341370	*A. marginale*	NA	South China	Water buffalo
DQ341369	*A. marginale*	NA	South China	Gansu, China
AF414878	*A. marginale*	Isolate 2:3	Zimbabwe	NA
AF311303	*A. marginale*	Virginia	Virginia, USA	Bovine
AF414873	*A. marginale*	Strain Veld	South Africa	NA
HM538192	*A. marginale*	Clone 1	Suizhou, China	Water buffalo
AJ633050	*A. ovis*	Isolate Yuzhong	Gansu, China	Goat
AJ633049	*A. ovis*	Isolate Jingtai	Gansu, China	Goat
AF414870	*A. ovis*	Isolate OVI	South Africa	NA
AF309865	*A. ovis*	Strain Idaho	Idaho, USA	NA
AY262124	*A. ovis*	NA	Jingtsai, China	Sheep
EF587237	*A. ovis*	Isolate CGX	Guangxi, China	NA
EU439943	*A. platys*	Strain Gigio	Basilicata, Italy	Dog
EF139459	*A. platys*	NA	Bangkok, Thailand	Dog
HQ872465	*A. phagocytophilum*	Strain HB-SZ-HGA-S05	Suizhou, Hubei, China	Goat
HQ629925	*A. phagocytophilum*	Strain Est2540	Estonia	*Ixodes ricinus*
HQ872464	*A. phagocytophilum*	Strain HB-SZ-HGA-S04	Suizhou, Hubei, China	Goat
AY527214	*A. phagocytophilum*	Strain Strong	Sweden	Horse
AF156784	*A. platys*	Strain Gzh981	NA	NA
M82801	*A. platys*	NA	North America	Dog
AF399917	*A. platys*	Strain Venezuela-Lara	Venezuela	Dog
U27104	*Anaplasma* sp. GA	Isolate No 4	Atlanta, Georgia ,USA	White-tailed deer
U54806	*Anaplasma* sp.	Omatjenne	Namibia	*Hyalomma truncatum* tick
U54805	*Anaplasma* sp.	Strain Germishuys	South Africa	Sheep
U27103	*Anaplasma* sp. GA	Isolate No 2	Atlanta, Georgia, USA	White-tailed deer
AF318023	*Anaplasma* sp.	Strain Bom Pastor	Mozambique	Goat
U77389	*Anaplasma* sp.	Strain Swiss horse 1	Switzerland	Horse
U03776	*E. ruminantium*	Omatjenne	South Africa	Ruminant
AF069758	*E. ruminantium*	Mara 87/7	South Africa	Ruminant
X62432	*E. ruminantium*	Senegal	South Africa	Ruminant
U03777	*E. ruminantium*	Ball 3	South Africa	Ruminant
X61659	*E. ruminantium*	Crystal Springs	Zimbabwe	Ruminant
U11021	*R. rickettsii*	Strain Sawtooth	NA	*Dermacentor andersonii* tick

NA* = Not available

7. Nucleotide sequence accession numbers

The 16S rRNA gene sequences of the sequences identified in this study have been submitted to GenBank with accession numbers KC189837-KC189854.

8. Quantitative pCS20 real-time PCR for the specific detection of *E. ruminantium*

The pCS20 quantitative real-time PCR (qPCR) for the specific detection of *E. ruminantium* was performed as described by Steyn *et al*. (2008). Primers CowF forward (5'-CAA AAC TAG TAG AAA TTG CAC A-3') and CowR reverse (5'-TGC ATC TTG TGG TGG TAC-3') amplifed a 226 bp fragment of the conserved pCS20 region. A TaqMan probe, Cow™ (5' 6FAM TCC TCC ATC AAG ATA TAT AGC ACC TAT TA XT-PH-3'), was used which was positioned 12 bp from the forward primer (synthesized by TIB-Molbiol, Berlin, Germany). The LightCycler® FastStart DNA Master Hybridization probe kit (Roche Diagnostics) was used; each reaction mixture contained 2 µl of DNA, 4 mM $MgCl_2$, 0.5 µM of each primer, 0.4 µM probe, 1 U Uracil DNA N-Glycosylase (UNG) and 5 U enzyme (Roche) to a total volume of 20 µl. Water was used as negative control and pCS20 plasmid DNA (10 µg/µl) as a positive control. Thermal cycling with a primary incubation of 10 min at 40°C was followed by a denaturing for 10 min at 95°C, which was then subsequently followed by 38 cycles of denaturing at 95°C, 10 sec with a 20°C/s slope, annealing at 48°C, 10 sec with a 20°C/s slope and elongation at 58°C, 30 sec with a 20°C/s slope, and a final cooling step to 40°C. Fluorescence data was acquired at the end of the extension step of each cycle and the data was analyzed using the LightCycler® software-4.0 (Roche). A threshold cycle of 38 was selected as the detection limit of this assay.

CHAPTER 4

RESULTS

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1. Reverse Line Blot (RLB) hybridization assay results

The RLB results obtained from 200 buffalo blood samples collected from the Kruger National Park (KNP) and the Hluhluwe-iMfolozi Park demonstrated the presence of *Anaplasma, Ehrlichia, Theileria* and *Babesia* species either as single or as mixed infections. A representative RLB result is shown in Figure 4.1.

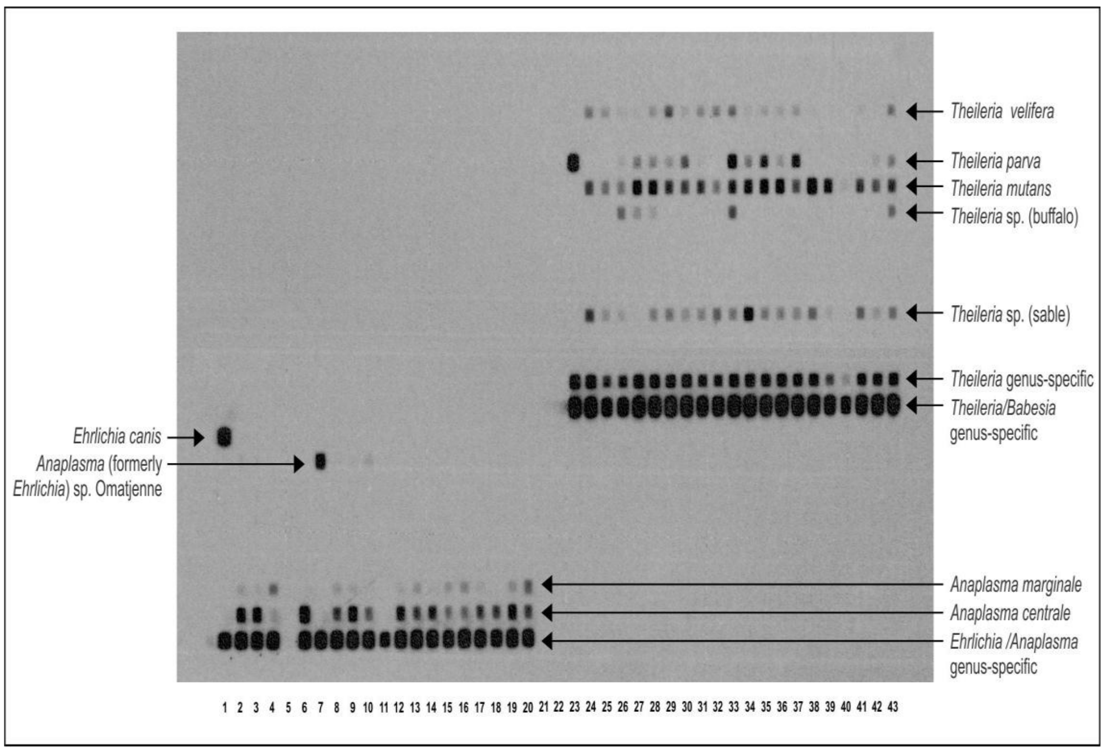

Figure 4.1: Representative reverse line blot (RLB) hybridization assay results for the simultaneous detection of *Ehrlichia, Anaplasma, Theileria* and *Babesia* species in the African buffalo blood samples.

1.1 *Anaplasma* and *Ehrlichia* species

Anaplasma species were detected either as single or as mixed infections (Table 4.1, Figure 4.2). Single infections were detected in 40% (n=40) of the samples from KNP and 34%

(n=34) of the samples from Hluhluwe-iMfolozi Park. Multiple infections with two or more species were found in 48% (n=48) of the samples from Hluhluwe-iMfolozi Park and 19% (n=19) of the samples from KNP. The most frequently occurring species detected from Hluhluwe-iMfolozi Park samples were: *A. centrale* (75%), *A. marginale* (42%) followed by *Anaplasma (Ehrlichia)* sp. Omatjenne (28%). The most frequently occurring species from the KNP were: *A. centrale* which ranged at 49% followed by *A. marginale* (24%) and *Anaplasma (Ehrlichia)* sp. Omatjenne (5%). The PCR product of 5% of the samples (n=5) from Hluhluwe-iMfolozi Park and 21% (n=21) of the samples from the KNP hybridized only with the *Ehrlichia/Anaplasma* genus-specific probes and not with any of the species-specific probes. The remaining 22% (n=22) of samples from KNP and 13% (n=13) from Hluhluwe-iMfolozi Park were negative (or below detection limit of the test) for any of the *Ehrlichia* and *Anaplasma* species.

Table 4.1: Occurrence of *Anaplasma* and *Ehrlichia* species infections in buffalo blood samples from the KNP and Hluhluwe-iMfolozi Park as determined by RLB hybridization.

	Kruger National Park (n = 100)	**Hluhluwe-iMfolozi Park (n = 100)**	**TOTAL (n = 200)**
Single infections:	**40 (40%)**	**34 (34%)**	**74 (37%)**
A. centrale	30	27	57 (28.5%)
A. marginale	5	3	8 (4%)
Anaplasma (Ehrlichia) sp. Omatjenne	5	4	9 (4.5%)
Mixed *Anaplasma* spp. infection:	**19 (19%)**	**48 (48%)**	**67 (33.5%)**
A. centrale	19	48	67 (33.5%)
A. marginale	19	39	48 (24%)
Anaplasma (Ehrlichia) sp. Omatjenne	0	24	24 (12%)
***Ehrlichia/Anaplasma* genus-specific only**	**19 (19%)**	**5 (5.0%)**	**24 (12%)**
Negative/below detection limit	**22 (22%)**	**13 (13.0%)**	**30 (17.5%)**

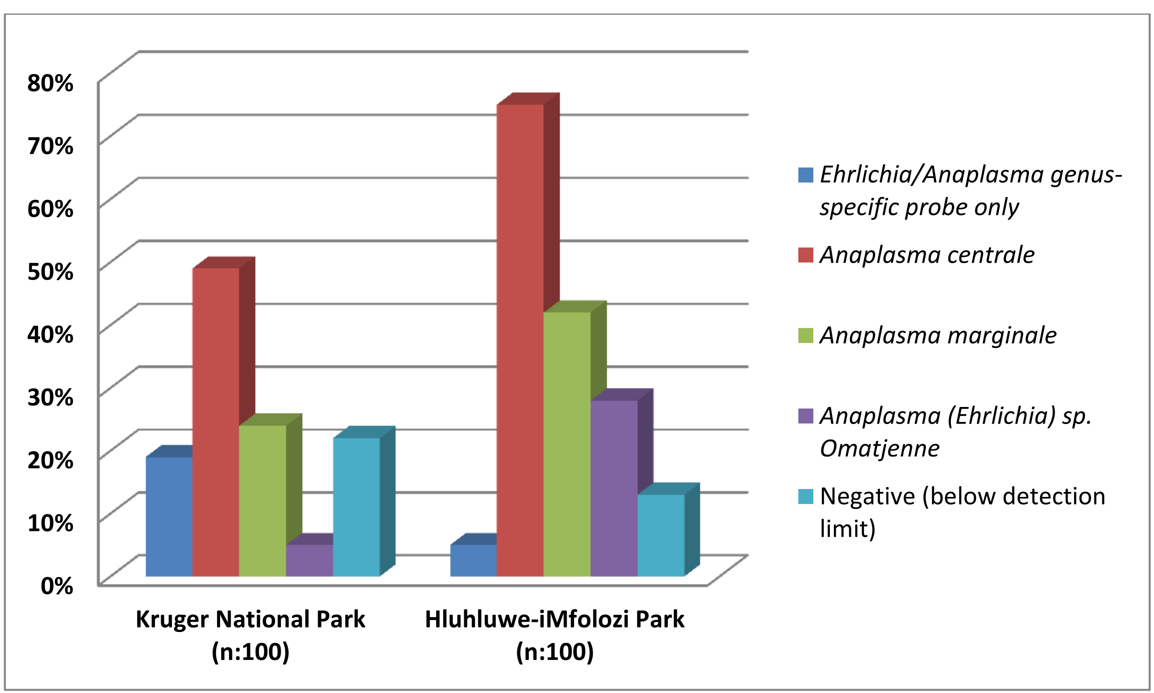

Figure 4.2: Occurrence of *Anaplasma* and *Ehrlichia* species in buffalo blood samples from the Hluhluwe-iMfolozi Park and KNP, South Africa as determined by the RLB hybridization assay.

1.2 *Theileria* and *Babesia* species.

Theileria and *Babesia* species were detected either as single or as mixed infections (Table 4.2, Figure 4.3). Single infections were found in 27% (n= 27) of the samples from KNP; none of the samples from the Hluhluwe-iMfolozi Park had single infections. Multiple infections with two or more species were found in 89% (n=89) of the samples from Hluhluwe-iMfolozi Park and 64% (n=64) of the sample from KNP. The most frequently occurring species detected in the KNP were: *T. mutans* (81%), *Theileria* sp. (sable) (61%), *T. parva* (40%), *Theileria* sp. (buffalo) (13%) and *T. velifera* (11%). In the Hluhluwe-iMfolozi Park, the most occurring species were *T. mutans* (55%), *T. velifera* (54%), *T. parva* (53%), *Theileria* sp. (sable) (53%), *Theileria* sp. (buffalo) (49%), *T. buffeli,* (49%) and *Babesia occultans* (50%), respectively. In 9% (n=9) of the KNP samples, the PCR products hybridized only with the *Theileria/Babesia* genus-specific probes. The remaining 11% of the samples from Hluhluwe-iMfolozi Park tested negative (or below detection limit) for any *Theileria* and *Babesia* species.

Table 4.2: Occurrence of *Theileria* and *Babesia* species infections in buffalo blood samples from the Kruger National Park and Hluhluwe-iMfolozi Park of South Africa as determined by RLB.

	Kruger National Park (n = 100)	**Hluhluwe-iMfolozi Park (n = 100)**	**TOTAL (n = 200)**
Single infections:	**27(27%)**	**0 (0%)**	**27 (13.5%)**
T. mutans	17	0	17 (8.5%)
T. parva	7	0	7 (3.5%)
Theileria sp. (buffalo)	3	0	3 (1.5)
Mixed *Theileria/Babesia* spp. infection:	**64 (64%)**	**89 (89%)**	**153 (76.5%)**
T. mutans	81	55	136 (68%)
Theileria sp. (sable)	61	53	114 (57%)
T. parva	40	53	93 (46.5%)
Theileria sp. (buffalo)	13	49	62 (31%)
T. velifera	11	54	65 (32.5%)
T. buffeli	0	49	49 (24.5%)
B. occultans	0	50	50 (25%)
***Theileria/Babesia* genus-specific only**	**9 (9%)**	**0 (0%)**	**9 (4.5%)**
Negative/below detection limit	**0 (0%)**	**11(11%)**	**11 (5.5%)**

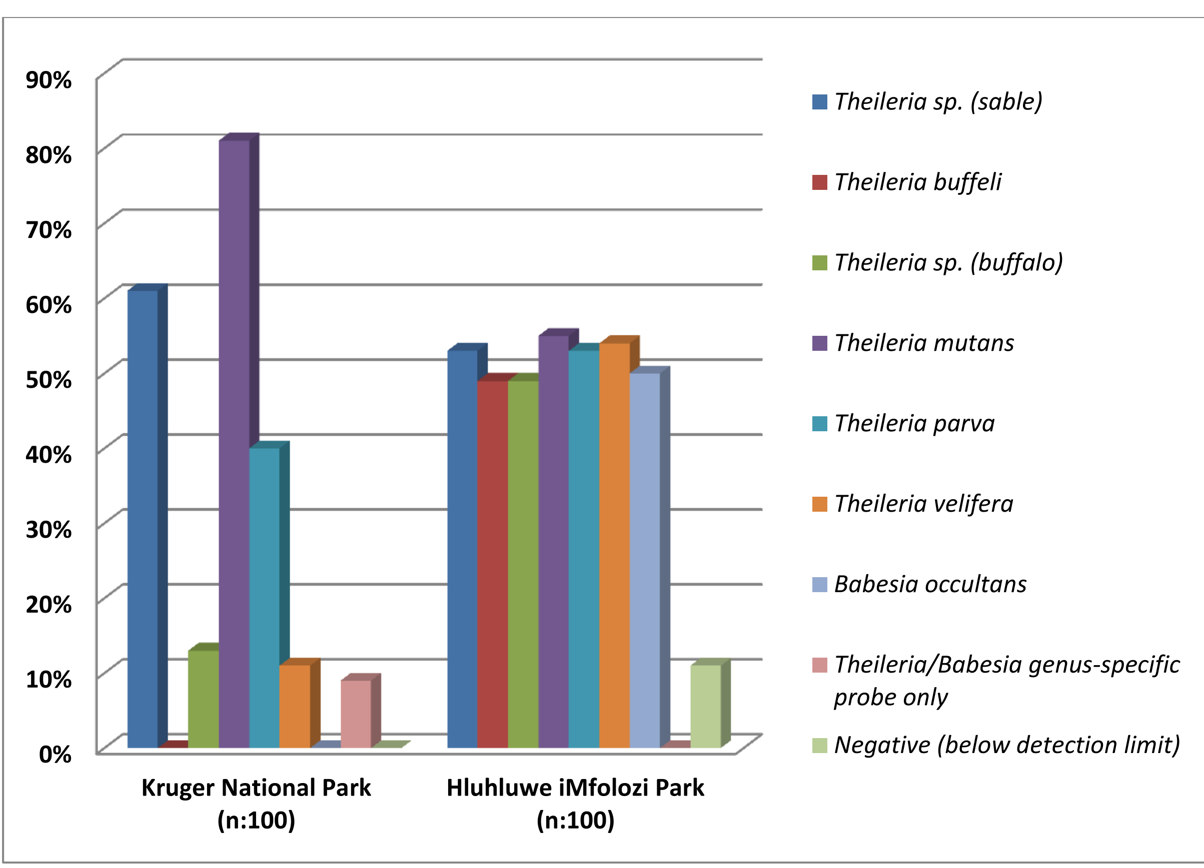

Figure 4.3: Occurrence of *Theileria* and *Babesia* species in buffalo blood samples from the Hluhluwe-iMfolozi Park and KNP, South Africa as determined by the RLB hybridization assay.

2. Quantitative real-time PCR for *Ehrlichia ruminantium*

As we did not detect any *Ehrlichia ruminantium* in any of the buffalo blood samples using the RLB assay, we subjected all samples to the *E. ruminantium* pCS20 real-time PCR assay. *Ehrlichia ruminantium* was detected in 5% (n=5) of the buffalo blood samples from the KNP and the remaining 95% (n=95) samples were negative (or below detection limit of the test). All the buffalo blood samples from Hluhluwe-iMfolozi Park (n=100) tested negative (or below detection limit of the test) for *Ehrlichia ruminantium* using the quantitative real-time PCR assay. A representative amplification curve is shown in Figure 4.4.

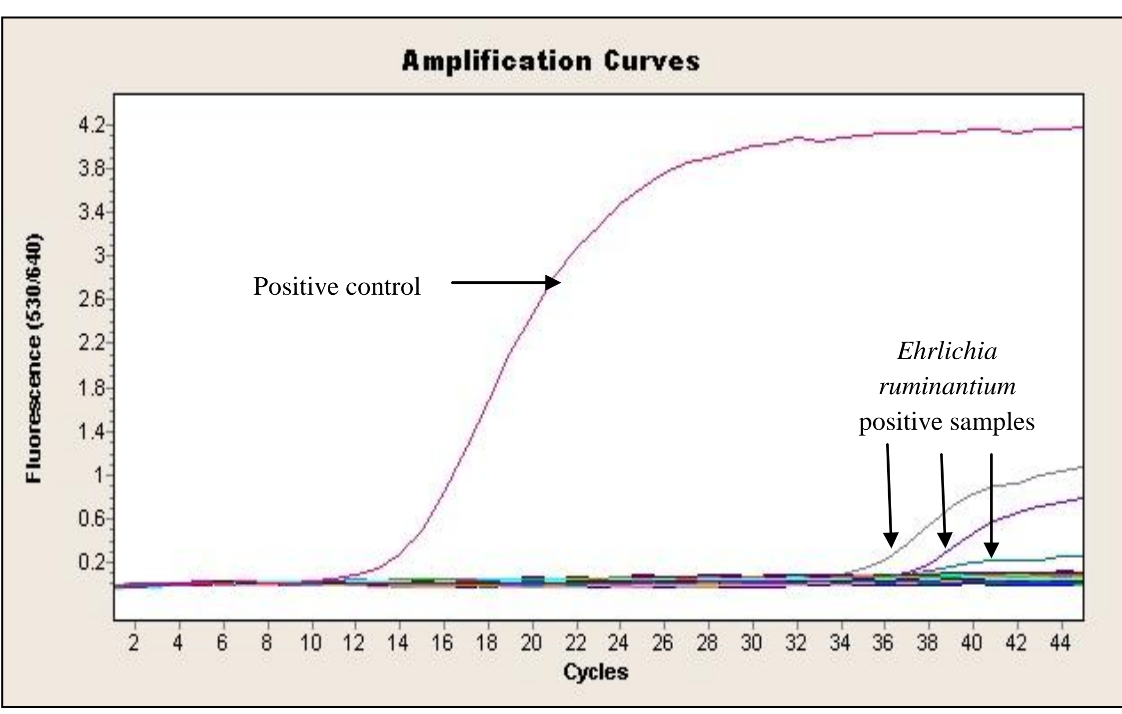

Figure 4.4: The amplification curve for detection of *Ehrlichia ruminatium* using the pCS20 coding gene in African buffalo blood samples**.**

For 36 of the samples, *T. parva* specific real-time PCR results were available from a separate study (Chaisi, *et al*., 2011) and were subsequently compared with the RLB results obtained in this study (Table 4.3). Twenty five (69.4%) of these samples tested positive for the presence of *T. parva* using the RLB assay compared to 31 (86.1%) samples using the real-time PCR assay. One of the samples (B10) tested positive for *T. parva* using the RLB assay while it tested negative using the real-time PCR assay.

Table 4.3: Comparison of RLB results obtained in this study with available *T. parva*-specific real-time PCR results. Shaded in grey: tested negative for *T. parva* on RLB assay but positive for *T. parva* with qPCR assay.

Sample no	RLB result	qPCR
A2	Negative*	-*
A4	*Theileria* sp. (sable), *T. buffeli*, ***T. parva***, *Theileria* sp. (buffalo), *T. mutans, B. occultans, T. velifera*	+
A5	*Theileria* sp. (buffalo), *T. mutans*	+
A6	*T. buffeli*, ***T. parva***, *Theileria* sp. (buffalo), *T. mutans, B. occultans*	+
A11	*T. buffeli, Theileria* sp. (buffalo)	-
A12	*T. buffeli, Theileria* sp. (buffalo)	-
A13	*Theileria* sp. (sable), *T. buffeli*, ***T. parva***, *Theileria* sp. (buffalo), *T. mutans, B. occultans, T. velifera*	+
A14	*Theileria* sp. (sable), ***T. parva***, *Theileria* sp. (buffalo), *T. mutans, B. occultans, T. velifera*	+
A15	*Theileria* sp. (sable), ***T. parva***, *Theileria* sp. (buffalo), *T. mutans, B. occultans, T. velifera*	+
A17	*Theileria* sp. (sable), ***T. parva***, *Theileria* sp. (buffalo), *T. mutans, B. occultans, T. velifera*	+
A18	*Theileria* sp. (sable), ***T. parva***, *Theileria* sp. (buffalo), *T. mutans, B. occultans, T. velifera*	+
A19	Negative*	-*
A25	*Theileria* sp. (sable), ***T. parva***, *Theileria* sp. (buffalo), *T. mutans, B.occultans, T. velifera*	+
A27	*Theileria* sp. (sable), ***T. parva***, *Theileria* sp. (buffalo), *T. mutans, B.occultans, T. velifera*	+
A28	Negative	+
A29	*T. buffeli, Theileria* sp. (buffalo)	+
A30	*T. buffeli*	+
A36	*Theileria* sp. (sable), ***T. parva***, *Theileria* sp. (buffalo), *T. mutans, B. occultans, T. velifera*	+
A38	*Theileria* sp. (sable), *T. buffeli*, ***T. parva***. *Theileria* sp. (buffalo), *T. mutans, B. occultans, T. velifera*	+
B1	*Theileria* sp. (sable), ***T. parva***, *Theileria* sp. (buffalo), *T. mutans, B. occultans, T. velifera*	+
B2	*Theileria* sp. (sable), ***T. parva***, *Theileria* sp. (buffalo), *T. mutans, B. occultans, T. velifera*	+
B3	*Theileria* sp. (sable), ***T. parva***, *Theileria* sp. (buffalo), *T. mutans, B. occultans, T. velifera*	+
B4	*Theileria* sp. (sable), ***T. parva***, *Theileria* sp. (buffalo), *T. mutans, B. occultans, T. velifera*	+
B5	*Theileria* sp. (sable), ***T. parva***, *Theileria* sp. (buffalo), *T. mutans, B. occultans, T. velifera*	+
B6	Negative	+
B9	*Theileria* sp. (sable), ***T. parva***, *Theileria* sp. (buffalo), *T. mutans, B. occultans, T. velifera*	+
B10	*Theileria* sp. (sable), ***T. parva***, *Theileria* sp. (buffalo), *T. mutans, B. occultans, T. velifera*	-
B13	*T.* sp. (sable), ***T. parva***, *T.* sp. (buffalo), *T. mutans, , T. velifera*	+
B14	Negative	+

B16	*Theileria* sp. (sable), ***T. parva***, *Theileria* sp. (buffalo), *T. mutans, B. occultans, T. velifera*	+
B17	*Theileria* sp. (sable), ***T. parva***, *Theileria* sp. (buffalo), *T. mutans, B. occultans, T. velifera*	+
B18	*Theileria* sp. (sable), ***T. parva***, *Theileria* sp. (buffalo), *T. mutans, B. occultans, T. velifera*	+
B19	*Theileria* sp. (sable), ***T. parva***, *Theileria* sp. (buffalo), *T. mutans, B. occultans, T. velifera*	+
B21	*Theileria* sp. (sable), ***T. parva***, *Theileria* sp. (buffalo), *T. mutans, B. occultans, T. velifera*	+
B22	Negative	+
B62	*Theileria* sp. (sable), *T. buffeli*, ***T. parva***, *Theileria* sp. (buffalo), *T. mutans, B. occultans, T. velifera*	+

*** :** Or below detection limit of the test

3. Cloning, sequencing and phylogenic analysis

A total of 33 samples from the KNP and 31 from the Hluhluwe-iMfolozi Park were selected for molecular characterization by cloning and sequencing of the 16S rRNA gene. These were selected on the basis of (i) either testing positive for a specific *Anaplasma* and/or *Ehrlichia* spp. (*A. centrale, A. marginale* and/or *Anaplasma* (*Ehrlichia*) sp. Omatjenne) using the RLB assay, or (ii) for only hybridizing with the *Anaplasma/Ehrlichia* genus-specific probes used. Although 28% of the samples collected from the Hluhluwe-iMfolozi Park tested positive for the presence of *Anaplasma* (*Ehrlichia*) sp. Omatjenne using the RLB assay, amplification of the full-length parasite 16S rRNA gene was unsuccessful; most probably due to low parasitaemia. This was also the case with the 5 samples from the KNP that previously tested positive for *E. ruminantium* using the pCS20 real-time PCR assay.

The amplification of the near full-length (~ 1306 bp) 16S rRNA gene or a partial (~ 726 bp) 16S rRNA gene from seven samples from the KNP and three from Hluhluwe-iMfolozi Park was successful. Five of these gave specific RLB results whereas the remainder hybridized with only the *Anaplasma/Ehrlichia* genus-specific probes used (Table 4.4). The PCR products were cloned and the recombinants were sequenced. Sequences were assembled, edited and aligned with sequences of related genera from Genbank (Table 4.4).

BLASTn homology searches indicated that the obtained sequences of 12 of the 18 clones were most similar to published *A. centrale* 16S rRNA gene sequences (accession numbers; AF309869, AF318944; AF414868, AF414869; EF520686, EF520687, EF520688, EF520689 and EF520690). Four clones were most similar to the published *A. marginale* sequence (AF311303, DQ341369, DQ341370 and HM538192), three of which were previously

identified from water buffalo in south China. The sequences of the remaining two clones were closely similar to *Anaplasma* sp. strain Omatjenne (previously *Ehrlichia* sp. strain Omatjenne) (ESU54806) (previously identified from goats and *Hyalomma truncatum* ticks in Namibia) and *Anaplasma platys* (AF399917 and EF139459) (previously identified from dogs in Venezuela and Thailand, respectively).

Table 4.4: Origin and results of the samples selected for 16S rRNA characterization by cloning and sequencing.

Sample no	Place of Origin	RLB results	Clone no	Sequence length (bp)	Phylogenetic classification
KNP/G13	*KNP	*A. centrale*	KNP/G13/a	726	*A. marginale*
KNP/G26	KNP	*Ehrlichia/Anaplasma* genus-specific	KNP/G26/a	726	*A. centrale*
			KNP/G26/b	726	*A. centrale*
KNP/I20	KNP	*Ehrlichia/Anaplasma* genus-specific	KNP/I20/a	1306	*A. centrale*
			KNP/I20/b	1306	*A. centrale*
KNP/K7	KNP	*Ehrlichia/Anaplasma* genus-specific	KNP/K7/a	726	*A. centrale*
KNP/M7	KNP	*A. centrale*	KNP/M7/a	726	*A. marginale*
KNP/M8	KNP	*Ehrlichia/Anaplasma* genus-specific	KNP/M8/a	726	*A. marginale*
KNP/M12	KNP	*A. marginale, A. centrale*	KNP/M12/a	726	*A. centrale*
			KNP/M12/b	726	*A. marginale*
HIP/A8	#HIP	*A. centrale*	HIP/A8/a	1306	*A. centrale*
			HIP/A8/b	1306	*Anaplasma (Ehrlichia)* sp. Omatjenne
			HIP/A8/c	1306	*A. centrale*
			HIP/A8/d	1306	*A. centrale*
			HIP/A8/e	1306	*A. centrale*
HIP/B17	HIP	*A. centrale, Anaplasma (Ehrlichia)* sp. Omatjenne	HIP/B17/a	726	*Anaplasma (Ehrlichia)* sp. Omatjenne
HIP/A11	HIP	*Ehrlichia/Anaplasma* genus-specific	HIP/A11/a	1306	*A. centrale*
			HIP/A11/b	1306	*A. centrale*

Kruger national Park-**KNP***

Hluhluwe-iMfolozi Park-**HIP**#

Estimated evolutionary divergences between the obtained gene sequences and related *Anaplasma* and *Ehrlichia* 16S rRNA gene sequences were subsequently compared by determining the number of base differences per sequence (Table 4.5 and 4.6). Some sequences were identical, whereas others differed from each other by one to six nucleotides. Comparisons of the novel sequences (n = 8) to published *A. centrale* 16S rRNA sequences over a region of 1306 bp (Table 4.5) indicated that HIP/A8/c was 100% identical to the *A. centrale* AF309869, AF318944; AF414868, AF414869; EF520686, EF520687, EF520688, EF520689 and EF520690. The novel *A. centrale* sequences obtained differed from each other with one to five nucleotides. The *A. centrale* KNP/I20/b and HIP/A8/e sequences were 100% identical over the 1305 bp region. When comparing the sequences over a 726 bp region, the novel *A. centrale* sequences (n = 12) differed from the published *A. centrale* sequences by one to nine nucleotides (Table 4.6). Partial sequences of KNP/I20/a and HIP/A11/a were 100% identical over the 726 bp region.

Sequence HIP/A8/b differed by four nucleotides from *Anaplasma (Ehrlichia)* sp. Omatjenne (U54805) (as originally described) and by six nucleotides from *A. platys* (M82801) (type species). This would suggest that sequence HIP/A8/b represents a novel *Anaplasma (Ehrlichia)* sp. Omatjenne sequence. However, when only taking the partial sequence into account (Table 4.6), HIP/A8/b was identical to *A. platys* EU439943, EF139459, AF399917 and to the obtained HIP/B17/a over a 726 bp region which would have suggested that both these obtained sequences represented *A. platys*.

We have unfortunately only obtained partial *A. marginale* 16S rRNA gene sequences. When comparing the sequences over a 726 bp region, the novel *A. marginale* sequences (n = 4) differed from the published *A. marginale* sequences by two nucleotides (Table 4.6). They also differed by two nucleotides from each other.

The observed sequence similarities were confirmed by phylogenetic analyses. There was no significant difference in the topology of the phylogenetic trees. Neighbor-joining, maximum likelihood, maximum parsimony and Bayesian analyses were used to reveal the relationships between the obtained sequences and related *Ehrlichia* and *Anaplasma* species previously deposited in Genbank. Representative trees obtained by the neighbor-joining method are shown in Figures 4.5 and 4.6.

Both neighbor-joining trees based on (i) the partial 16S rRNA gene (726 bp) (Figure 4.5) and (ii) near-full length 16S rRNA gene (1306 bp) (Figure 4.6) revealed six groups representing *A. centrale*, *A. marginale*, *A. ovis*, *A. phagocytophilum*, *Anaplasma* (*Ehrlichia*) sp. strain Omatjenne / *A. platys* and *E. ruminantium*. Twelve of the obtained clone sequences clustered in the *A. centrale* group, four clustered in the *A. marginale* group and two clustered in the *Anaplasma* (*Ehrlichia*) sp. strain Omatjenne / *A. platys* group.

Table 4.5. Estimates of evolutionary divergence between sequences. The number of base differences per sequence from analysis between sequences is shown. All results are based on the pairwise analysis of 32 sequences. Analyses were conducted in MEGA4 (Tamura, *et al.,* 2007). All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons (Pairwise deletion option). There were a total of 1306 positions in the final dataset.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
1	*E. ruminantium* (U03777)																																
2	*E. ruminantium* (X62432)	0																															
3	*E. ruminantium* (X61659)	0	0																														
4	*E. ruminantium* (AF069758)	2	2	2																													
5	*E. ruminantium* (U03776)	1	1	1	3																												
6	*Ehrlichia* sp. (U54805)	30	30	30	32	31																											
7	KNP/I20/b	101	101	101	103	100	98																										
8	HIP/A8/e	101	101	101	103	100	98	0																									
9	HIP/A8/a	101	101	101	103	100	98	2	2																								
10	HIP/A8/d	101	101	101	104	101	99	3	3	3																							
11	HIP/A11/b	102	102	102	103	100	98	2	2	2	3																						
12	HIP/A8/c	101	101	101	102	99	97	1	1	1	2	1																					
13	*A. centrale* (AF318944)	100	100	100	102	99	97	1	1	1	2	1	0																				
14	*A. centrale* (AF309869)	100	100	100	102	99	97	1	1	1	2	1	0	0																			
15	*A. centrale* (EF520686)	100	100	100	102	99	97	1	1	1	2	1	0	0	0																		
16	*A. centrale* (EF520689)	100	100	100	102	99	97	1	1	1	2	1	0	0	0	0																	
17	*A. centrale* (AF414869)	100	100	100	102	99	97	1	1	1	2	1	0	0	0	0	0																
18	*A. centrale* (EF520687)	100	100	100	102	99	97	1	1	1	2	1	0	0	0	0	0	0															
19	*A. centrale* (EF520690)	100	100	100	102	99	97	1	1	1	2	1	0	0	0	0	0	0	0														
20	*A. centrale* (EF520688)	100	100	100	102	99	97	1	1	1	2	1	0	0	0	0	0	0	0	0													
21	*A. centrale* (AF414868)	101	101	101	103	100	98	2	2	2	3	2	1	1	1	1	1	1	1	1	1												
22	HIP/A11/a	101	101	101	103	100	98	4	4	4	5	4	3	3	3	3	3	3	3	3	3	4											
23	KNP/I20/a	100	100	100	102	99	97	3	3	3	4	3	2	2	2	2	2	2	2	2	2	3	3										
24	*A. platys* (EU439943)	98	98	98	100	97	101	39	39	39	40	39	38	38	38	38	38	38	38	38	38	39	39	38									
25	*A. platys* (EF139459)	98	98	98	100	97	101	39	39	39	40	39	38	38	38	38	38	38	38	38	38	39	39	38	0								
26	*A. platys* (AF156784)	98	98	98	100	97	101	39	39	39	40	39	38	38	38	38	38	38	38	38	38	39	39	38	0	0							
27	*A. platys* (M82801)	101	101	101	103	100	103	41	41	41	42	41	40	40	40	40	40	40	40	40	40	41	41	40	3	3	3						
28	*A. platys* (AF399917)	99	99	99	101	98	101	38	38	38	39	38	37	37	37	37	37	37	37	37	37	38	38	37	1	1	1	3					
29	*Anaplasma* sp. (ESU54806)	99	99	99	101	98	104	38	38	38	39	38	37	37	37	37	37	37	37	37	37	38	38	37	3	3	3	3	4				
30	*Anaplasma* sp. (AF318023)	98	98	98	100	97	103	37	37	37	38	37	36	36	36	36	36	36	36	36	36	37	37	36	2	2	2	5	3	1			
31	HIP/A8/b	99	99	99	101	98	101	38	38	38	39	38	37	37	37	37	37	37	37	37	37	38	38	37	3	3	3	6	4	4	3		
32	*Rickettsia rickettsii* (U11021)	205	205	205	205	204	211	206	206	206	207	206	205	205	205	205	205	205	205	205	205	204	204	205	211	211	211	213	210	214	213	212	

Table 4.6. Estimates of evolutionary divergence between sequences. The number of base differences per sequence from analysis between sequences is shown. All results are based on the pairwise analysis of 48 sequences. Analyses were conducted in MEGA4 (Tamura, *et al.*, 2007). There were a total of 726 positions in the final dataset.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48
1	*E. ruminantium* (U03777)																																																
2	*E. ruminantium* (X62432)	1																																															
3	*E. ruminantium* (X61659)	0	1																																														
4	*E. ruminantium* (AF069758)	0	1	0																																													
5	*E. ruminantium* (U03776)	1	2	1	1																																												
6	*Ehrlichia* sp. (U54805)	15	16	15	15	16																																											
7	*Anaplamsa* sp. (AF318023)	60	61	60	66	59	64																																										
8	*Anaplasma* sp. (U54806)	60	61	60	60	59	64	0																																									
9	*A. platys* (EU439943)	59	60	59	59	58	63	1	1																																								
10	*A. platys* (M82801)	61	62	61	61	60	65	3	2	2																																							
11	*A. platys* (EF139459)	59	60	59	59	58	63	1	1	0	2																																						
12	HIP/B17/a	59	60	59	59	58	63	1	1	0	2	0																																					
13	*A. platys* (AF399917)	59	60	59	59	58	63	1	1	0	2	0	0																																				
14	HIP/A8/b	59	60	59	59	58	63	1	1	0	2	0	0	0																																			
15	*A. platys* (AF156784)	59	60	59	59	58	63	1	1	0	2	0	0	0	0																																		
16	*Anaplasma* sp. (U77389)	59	55	54	54	53	58	9	9	8	10	8	8	8	8	8																																	
17	*A. phagocytophilum* (HQ872465)	56	57	56	56	55	60	9	9	8	10	8	8	8	8	8	2																																
18	KNP/I20/b	60	61	60	60	59	60	25	25	26	28	26	26	26	26	26	25	23																															
19	HIP/A8/e	60	61	60	60	59	60	25	25	26	28	26	26	26	26	26	25	23	0																														
20	HIP/A8/a	60	61	60	60	59	60	25	25	26	28	26	26	26	26	26	25	23	2	2																													
21	HIP/A11/b	60	61	60	60	59	60	25	25	26	28	26	26	26	26	26	25	23	2	2	2																												
22	HIP/A8/d	60	61	60	60	59	60	25	25	26	28	26	26	26	26	26	25	23	2	2	2	2																											
23	HIP/A11/a	60	61	60	60	59	60	25	25	26	28	26	26	26	26	26	25	23	2	2	2	2	2																										
24	KNP/I20/a	60	61	60	60	59	60	25	25	26	28	26	26	26	26	26	25	23	2	2	2	2	2	0																									
25	HIP/A8/b	59	60	59	59	58	59	24	24	25	27	25	25	25	25	25	24	22	1	1	1	1	1	1	1																								
26	*A. centrale* (AF309869)	59	60	59	59	58	59	24	24	25	27	25	25	25	25	25	24	22	1	1	1	1	1	1	1	0																							
27	*A. centrale* (AF318944)	59	60	59	59	58	59	24	24	25	27	25	25	25	25	25	24	22	1	1	1	1	1	1	1	0	0																						
28	*A. centrale* sp. (EF520687)	59	60	59	59	58	59	24	24	25	27	25	25	25	25	25	24	22	1	1	1	1	1	1	1	0	0	0																					
29	*A. centrale* sp. (EF520690)	59	60	59	59	58	59	24	24	25	27	25	25	25	25	25	24	22	1	1	1	1	1	1	1	0	0	0	0																				
30	*A. centrale* sp. (EF520688)	59	60	59	59	58	59	24	24	25	27	25	25	25	25	25	24	22	1	1	1	1	1	1	1	0	0	0	0	0																			
31	*A. centrale* (AF414869)	59	60	59	59	58	59	24	24	25	27	25	25	25	25	25	24	22	1	1	1	1	1	1	1	0	0	0	0	0	0																		
32	*A. centrale* (EF520686)	59	60	59	59	58	59	24	24	25	27	25	25	25	25	25	24	22	1	1	1	1	1	1	1	0	0	0	0	0	0	0																	
33	*A. centrale* (EF520689)	59	60	59	59	58	59	24	24	25	27	25	25	25	25	25	24	22	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0																
34	KNP/K7/a	63	64	63	63	62	63	28	28	29	31	29	29	29	29	29	28	26	5	5	5	5	5	5	5	4	4	4	4	4	4	4	4	4															
35	*A. centrale* (AF414868)	60	61	60	60	59	60	25	25	26	28	26	26	26	26	26	25	23	2	2	2	2	2	2	2	1	1	1	1	1	1	1	1	1	5														
36	KNP/G26/b	63	64	63	63	62	63	26	26	27	29	27	27	27	27	27	28	26	5	5	5	5	5	5	5	4	4	4	4	4	4	4	4	4	8	5													
37	KNP/M12/a	60	61	60	60	59	60	23	23	24	26	24	24	24	24	24	25	23	2	2	2	2	2	2	2	1	1	1	1	1	1	1	1	1	5	2	3												
38	KNP/G26/a	62	63	62	62	61	62	27	27	28	30	28	28	28	28	28	29	27	6	6	6	6	6	6	6	5	5	5	5	5	5	5	5	5	9	6	7	4											
39	*A. ovis* (EF587237)	55	56	55	55	54	57	20	20	21	23	21	21	21	21	21	20	18	5	5	5	5	5	5	5	4	4	4	4	4	4	4	4	4	8	5	8	5	9										
40	*A. marginale* (DQ341370)	54	55	54	54	53	55	22	22	23	25	23	23	23	23	23	22	20	7	7	7	7	7	7	7	6	6	6	6	6	6	6	6	6	10	7	10	7	9	2									
41	*A. marginale* (DQ341369)	54	55	54	54	53	55	22	22	23	25	23	23	23	23	23	22	20	7	7	7	7	7	7	7	6	6	6	6	6	6	6	6	6	10	7	10	7	9	2	0								
42	*A. marginale* (HM538192)	54	55	54	54	53	55	22	22	23	25	23	23	23	23	23	22	20	7	7	7	7	7	7	7	6	6	6	6	6	6	6	6	6	10	7	10	7	9	2	0	0							
43	*A. marginale* (AF311303)	54	55	54	54	53	55	22	22	23	25	23	23	23	23	23	22	20	7	7	7	7	7	7	7	6	6	6	6	6	6	6	6	6	10	7	10	7	9	2	0	0	0						
44	KNP/M12/b	56	57	56	56	55	57	22	22	23	25	23	23	23	23	23	24	22	9	9	9	9	9	9	9	8	8	8	8	8	8	8	8	8	12	9	10	7	9	4	2	2	2	2					
45	KNP/G13/a	56	57	56	56	55	57	22	22	23	25	23	23	23	23	23	24	22	9	9	9	9	9	9	9	8	8	8	8	8	8	8	8	8	12	9	10	7	9	4	2	2	2	2	2				
46	KNP/M8/a	55	56	55	55	54	56	22	22	22	24	22	22	22	22	22	23	21	9	9	9	9	9	9	9	8	8	8	8	8	8	8	8	8	12	9	10	7	9	4	2	2	2	2	2	2			
47	KNP/M7/a	56	57	56	56	55	57	22	22	23	25	23	23	23	23	23	24	22	9	9	9	9	9	9	9	8	8	8	8	8	8	8	8	8	12	9	10	7	9	4	2	2	2	2	2	2	2		
48	*Rickettsia rickettsii* (U11021)	122	123	122	122	121	124	123	123	122	124	122	122	122	122	122	117	118	119	119	119	119	119	119	119	118	118	118	118	118	118	118	118	118	120	117	122	119	119	119	119	119	119	119	121	121	120	119	

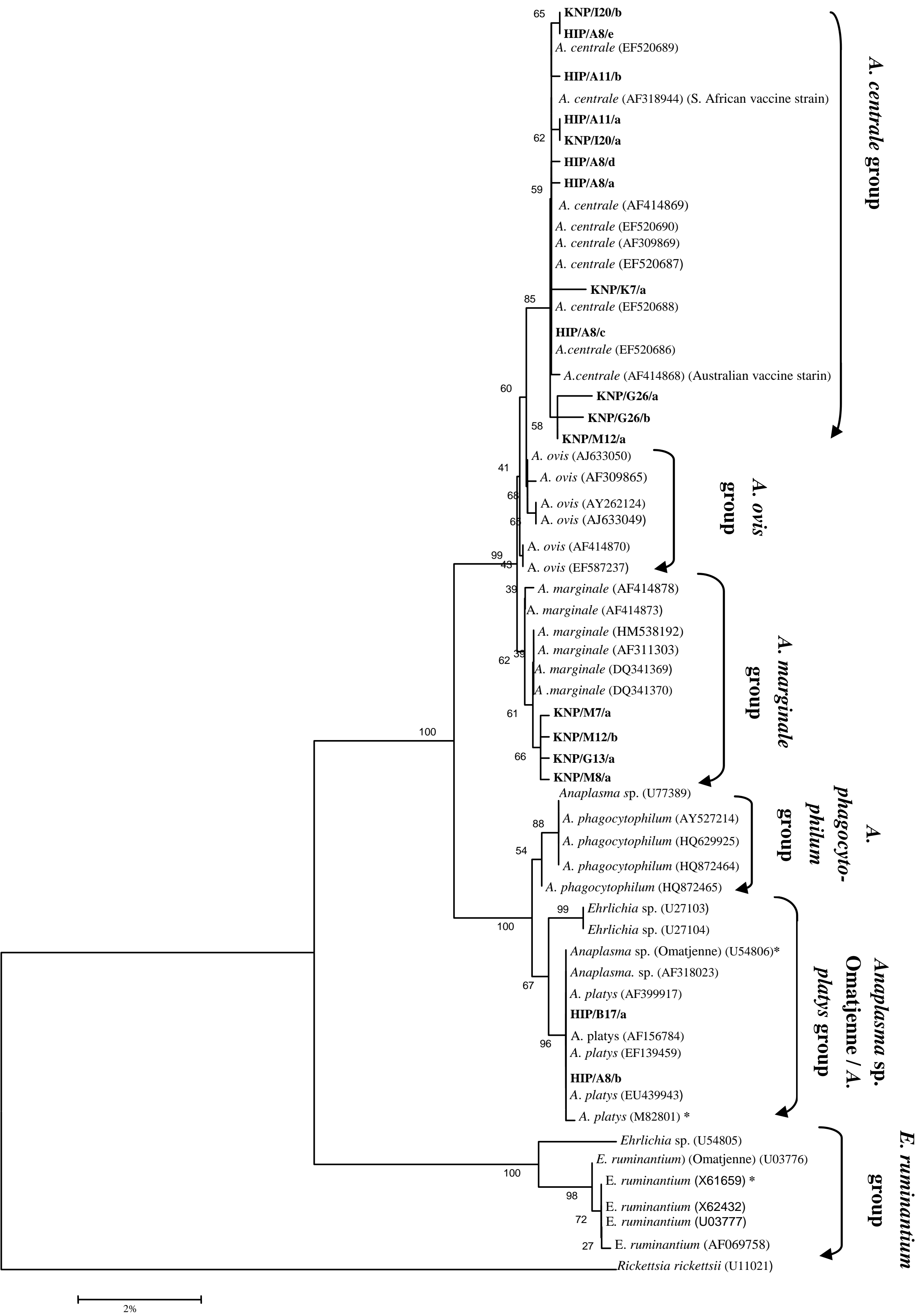

Figure 4.5: Neighbor-joining phylogenetic tree of all the 16S RNA gene sequences of *Ehrlichia* and *Anaplasma* species generated from the study together with related sequences from Genbank. The length of the alignment was truncated to the size of the smallest sequence which in this case was 726 bp. The accession numbers of the sequences from Genbank are indicated at the beginning of the name of each sequence. The names of the sequences generated in the study start with letters HIP and KNP.

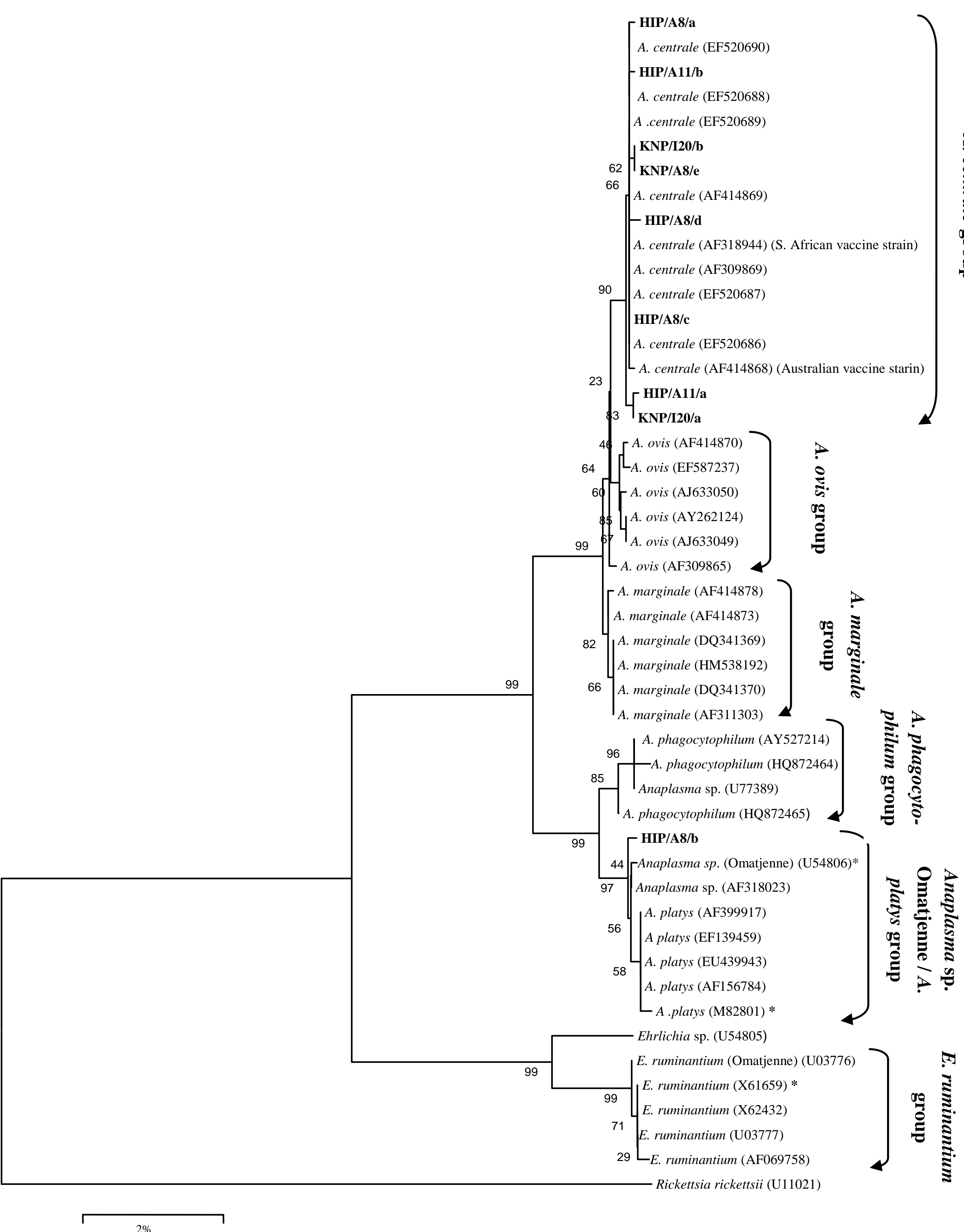

Figure 4.6: Neighbor-joining phylogenetic tree of 1306 bp of the 16S RNA gene of *Ehrlichia* and *Anaplasma* species generated from the study aligned together with related sequences from Genbank. In this tree the sequences alignment was truncated to the size of the smallest sequence which in this case was 1306 bp. The accession numbers of the sequences from Genbank are indicated at the beginning of the name of each sequence. The names of the sequences generated in the study start with letters HIP and KNP .

4. RLB probe development

In an attempt to design new RLB probes to detect the novel 16S rRNA gene sequences found in our study, the V1 hypervariable region of the 16S rRNA gene of published *A. centrale*, *A. marginale* and *Anaplasma (Ehrlichia)* sp. Omatjenne gene sequences were aligned with the novel sequences. A 100 bp region of the alignment including the area from which the RLB oligonucleotides were developed is shown in Figure 4.7; however the *A. marginale* sequences were too short to show the areas were the RLB probe hybridized. Unfortunately, no area could be found that displayed enough sequence variation. It was however, shown that the current RLB probe used in the assay is conserved amongst the novel sequences found, suggesting that all novel variants should be detected by the RLB hybridization assay.

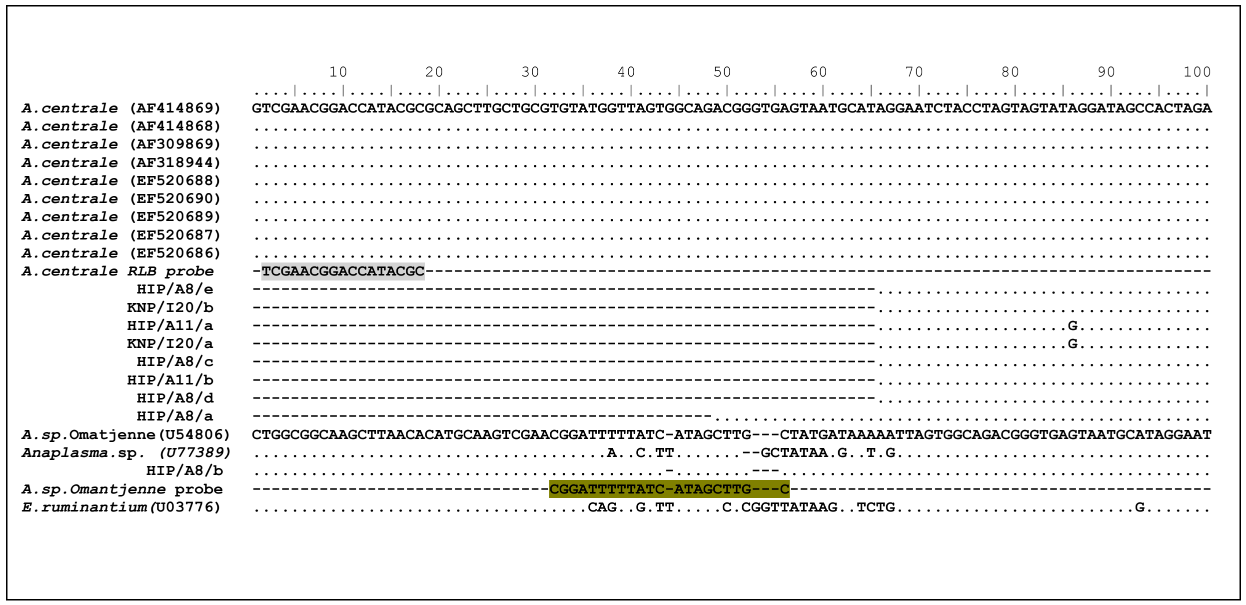

Figure 4.7: Nucleotide alignment of a 100 bp region each of the V1 hypervariable region of the published 16S rRNA gene of *A. centrale, Anaplasma (Ehrlichia)* sp. Omatjenne as well as the variants identified in the study. The *A. centrale* RLB probe sequence is highlighted in grey and the *Anaplasma (Ehrlichia)* sp. Omatjenne probe in green.

CHAPTER 5

DISCUSSION

The African buffalo is an important reservoir for various tick-borne haemoparasites of veterinary importance. In this study, the occurrence of tick-borne haemoprotozoan parasites (*Theileria*, *Babesia, Anaplasma* and *Ehrlichia* species) in buffalo from two geographically isolated national parks in South Africa (Kruger National Park and Hluhluwe-iMfolozi Park) was determined using the reverse line blot (RLB) hybridization assay. The RLB results revealed the presence of *Theileria*, *Babesia* and *Anaplasma* species either as single or as mixed infections. Although not detected with the RLB assay, 5% of the buffalo blood samples from the KNP tested positive for the presence of *Ehrlichia ruminantium* using the pCS20 real-time PCR assay (Steyn, *et al*., 2008). Previous studies on the occurrence of haemoparasites in the South African buffalo population have mainly focussed on the prevalence of *Theileria* species only. The finding on the presence of also *Anaplasma, Ehrlichia* and *Babesia* species is therefore a novel contribution.

This study has confirmed the findings of previous studies that buffalo is commonly co-infected with different *Theileria* spp., namely, *T. parva*, *Theileria* sp. (buffalo), *T. mutans*, *T. velifera* and *T. buffeli* (Allsopp, *et al*., 1993; Gubbels, *et al.*, 1999; Oura, *et al*., 2004; Pienaar, *et al*., 2011; Chaisi, *et al*., 2011). It was thus not an unexpected finding as it is well-known that the African buffalo is the natural reservoir host of both pathogenic and non-pathogenic *Theileria* species (Young, *et al*., 1977; Uilenberg, 1995). In this study, the most frequently occurring *Theileria* species detected in the KNP were *T. mutans*, *Theileria* sp. (sable), *T. parva*, *Theileria* sp. (buffalo) and *T. velifera*. *Theileria buffeli* was not detected in the KNP which was in concordance with the findings of Chaisi *et al*. (2011). In the Hluhluwe-iMfolozi Park, the most frequently occurring *Theileria* species were *T. mutans*, *T. velifera*, *T. parva*, *Theileria* sp. (sable), *Theileria* sp. (buffalo) and *T. buffeli*. In the study by Chaisi *et al*. (2011), the authors found that *Theileria mutans* and *T. parva* were the most prevalent *Theileria* spp. present in buffalo from the KNP, while *T. buffeli* was the most prevalent

Theileria species in the Hluhluwe-iMfolozi Park, followed by *Theileria* sp. (buffalo) and *T. parva.* The results obtained in this study compared well with those of Chaisi *et al*. (2011), except that in our study we also found *Theileria* sp. (sable) to be present in buffalo from both parks. In the study done by Pienaar *et al*. (2011), buffalo samples from the Marakele National Park and KNP were screened for *Theileria* and *Babesia* species using the RLB assay. The samples were also screened specifically for *T. parva* using the *T. parva*-specific real-time PCR assay (Sibeko, *et al*., 2008). RLB results indicated the presence of *T. parva, Theileria* sp. (buffalo), *T. mutans* and *T. velifera* in both parks. *Theileria buffeli* was only present in the Marakele National Park samples (~9%), while they also did not detect *T. buffeli* in any of the KNP samples. The RLB assay detected *T. parva* in ~40% of the samples, while ~70% of the samples tested positive for *T. parva* using the real-time PCR assay. This was not unexpected as other researchers have shown that qPCR is a more sensitive technique than RLB. Bhoora *et al*. (2010) also demonstrated that qPCR is more sensitive than the RLB hybridization assay and that *Theileria* and *Babesia* spp. infections can be detected by real-time PCR in samples that test negative by the RLB hybridization assay. *Theileria parva* specific qPCR results of 36 of the samples were available from a separate study (Chaisi, *et al*., 2011). When compared to the RLB results, similar results weer obtained as those by Pienaar *et al*. (2011) in that ~70% of these samples tested positive for the presence of *T. parva* using the RLB assay compared to ~86% samples using the qPCR assay.

Theileria sp. (sable) causes fatal clinical disease in roan (*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 previously been identified from healthy animals, such as African buffalo in South Africa, African short-horn cattle in Tanzania, blesbok in Swaziland as well as from blue wildebeest, klipspringer and common reedbuck in South Africa (Nijhof, *et al*. 2005). It was also identified in nyala (*Tragelaphus angasii*) from four game ranches in the Pongola area, northern KwaZulu-Natal (Pfitzer, *et al*., 2011). The main vectors are possibly *Rhipicephalus evertsi evertsi* and *R. appendiculatus* (Nijhof, *et al.* 2005). Based on the findings of Nijhof *et al*. (2005) and Pfitzer *et al*. (2011), it can only be speculated that this is also very likely to be a true finding of *Theileria* sp. (sable) in the buffalo population in KNP and Hluhluwe-iMfolozi Park

Babesia occultans was detected in 50% of the buffalo from the Hluhluwe-iMfolozi Park. *Babesia occultans* n. sp. (Gray & De Vos, 1981), the causative agent of a benign form of cattle babesiosis in South Africa, was originally obtained from a farm in the Northern Transvaal (now Limpopo Province), South Africa, in 1976 (Thomas & Mason, 1981). It causes a mild disease in cattle and is transmitted transovarially by *Hyalomma marginatum rufipes* (Gray & De Vos, 1981; Thomas & Mason, 1981). This species occurred at low parasitaemias and did not seem to cause significant clinical reactions in normal animals. The authors speculated that *B. occultans* originated from African antelope. To date, this organism has not been identified in wildlife in South Africa. In a recent study by Ros-García *et al.* (2011), *Babesia occultans* DNA was detected in *Hyalomma marginatum* unfed ticks collected in three bioclimatic regions in Tunisia, North Africa. Since *Babesia occultans* has been reported to only cause a mild disease in cattle, the possible tick tansmission of this parasite to the cattle crazing in close proximity to the Hluhluwe-iMfolozi Park fences should not be of great risk to the cattle population. The possible risk to antelope species remains unknown.

In 9% of the KNP buffalo blood samples, the PCR products hybridized only with the *Theileria/Babesia* genus-specific probe which could suggest the presence of a novel species or variant of a species. In the study by Chaisi *et al*. (2011), 22.5% of the KNP and 21% of Hluhluwe-iMfolozi Park buffalo samples hybridized with the *Theileria/Babesia* genus-specific probe only. The full-length 18S rRNA gene of selected samples was amplified, cloned and sequenced; in addition to the identification of 18S rRNA gene sequences that were similar to published *Theileria* spp. of cattle and buffalo, these authors also identified *Theileria* sp. (bougasvlei) and *T. sinensis*-like sequences, as well as novel 18S rRNA gene variants of *T. mutans*, *T. velifera* and *T. buffeli*. In a recent study, Mans *et al*. (2011) cloned and sequenced the V4 hypervariable region of the *Theileria* 18S rRNA gene from 62 buffalo and 49 cattle samples and identified 26 genotypes which included known and novel genotypes for the *T. buffeli, T. mutans, T. taurotragi* and *T. velifera* clades. If the parasite 18SrRNA gene of those PCR products that hybridized only with the *Theileria/Babesia* genus-specific probe have also been cloned and sequenced results surely would have contributed to the number of novel genotypes and/or variants found to date in the South African buffalo population.

Ehrlichia ruminantium has been reported to infect not only domesticated ruminants but also wild ruminants; however most wildlife species appear to carry the organism asymptomatically (Peter, *et al.* 2002). *Ehrlichia ruminantium* infects five domestic species namely cattle, sheep, goats, water buffalo and the domestic ferret (Mason & Alexander, 1940; Uilenberg, 1983). Data on *E. ruminantium* susceptibility in wild animals are diverse, both in terms of the species implicated as hosts and the reliability of different reports (Peter, *et al.*, 1998). The detection of *E. ruminantium* DNA in any of the buffalo samples tested using the RLB hybridization assay was unsuccessful. However, using the quantitative pCS20 real-time PCR assay (Steyn, *et al*., 2008), we detected *E. ruminantium* in 5% of the KNP samples. None of the Hluhluwe-iMfolozi Park samples tested positive for *E. ruminantium* using the real-time PCR assay.

To confirm these results, an attempt was made to clone and sequence the parasite 16S rRNA gene from the *E. ruminantium* qPCR positive samples. This was unsuccessful, however, probably due to low rickettsaemia. Since it is not known where *E. ruminantium* resides in carrier animals,it may be that the rickettsaemia may be very low in the blood of carriers, which would explain why we were not able to readily detect it using molecular tools. Low rickettsaemias were in fact revealed by the low real-time PCR cycle threshold (Ct value) of between 34 and 36 cycles. A threshold cycle of 38 was suggested by Steyn, *et al*. (2008) as the detection limit of this assay. The assay was previously shown to be able to detect seven copies of the *E. ruminantium* pCS20 sequence /ml of cell culture (Steyn, *et al*., 2008).

Results might suggest that buffalo are not a natural reservoir host of *E. ruminantium* and that other wildlife species need to be investigated as possible reservoir hosts. However, a subclinical carrier state in buffalo has been experimentally shown to occur after tick transmission from carrier animals (Peter, *et al*., 1998), thereforefurther studies will have to be done to confirm findings from this study. One should realize that if *E. ruminantium* were to become established in wildlife and vector populations, it could be impossible to eradicate. Without effective acaricide treatment, wildlife could sustain *Amblyomma* tick populations (where domestic animals are treated) and might be capable of maintaining a cycle of *E. ruminantium* transmission independently of domestic hosts (Allsopp, *et al*., 1999; Peter, *et al*., 1999).

Anaplasmosis is endemic in most cattle-farming areas of South Africa; *A. marginale* causes clinical bovine anaplasmosis and *A. centrale* only mild disease. The latter is being used as a live vaccine for cattle in South Africa. In this study, *A. centrale*, *A. marginale* and *Anaplasma (Ehrlichia)* sp. Omatjenne DNA were detected from Hluhluwe-iMfolozi Park samples as well as from KNP. Apart from cattle, *A. marginale* has previously been described in wild ruminants which can become persistently infected serving as reservoirs for infection of susceptible hosts (Kocan, *et al*., 2003). Serologic evidence of anaplasmosis in African buffalo has been observed (Kuttler, 1965). Brocklesby and Vidler (1966) observed *Anaplasma* bodies similar to *A. centrale* in buffalo erythrocytes, but on subinoculation no detectable infection occurred in splenectomized calves. A mild *A. marginale* isolate has been recovered from buffalo by inoculation of bovines (Potgieter, 1979). It is probable that buffalo would sustain *A. marginale* if splenectomized buffalo calves were inoculated, but no evidence confirming this is available.

In the study conducted by Oura *et al*. (2011), buffalo (n = 19) from Lake Mburo National Park (LMNP) in Uganda were found to be carriers of *T. parva, T. mutans, T. velifera, T. buffeli, Theileria* sp. (buffalo), *A. marginale* (74%) and *A. centrale* (63%) using the RLB assay, indicating that buffalo have the potential to spread these parasites to cattle. Impala were found to carry only *A. centrale* and the authors speculated that since *A. centrale* was not identified in cattle in Uganda (Oura, *et al*., 2004), it is possible that the *A. centrale*, found in both buffalo and impala, cannot be transmitted to cattle (Oura, *et al*., 2011). This could be true in South Africa as well, as *A. centrale* was identified in many of the buffalo samples in this study, however historically it has only been isolated from cattle a few times (Theiler, 1911; Potgieter, 1979). It is now wide-spread in cattle populations in South Africa since it is used in the live blood vaccine.

In a subsequent study, Oura *et al.* (2011) showed that 84% (n = 12) buffalo from Murchison Falls National Park (MFNP), 56% (n = 25) from Kidepo Valley National Park (KVNP) and 63% (n = 27) from Queen Elizabeth National Park (QENP) were carriers of *A. centrale*. *Anaplasma marginale* was detected in 50% buffalo form MFNP, 68% from KVNP and 40% from QENP. None of the sampled buffalo were carriers of *T. taurotragi*, *A. bovis, E. ruminantium, B. bovis* or *B. bigemina,* suggesting that these parasites do not circulate in buffalo or that a state of endemic instability exists for these parasites in buffalo in Uganda

(Oura, *et al*., 2011). This was in concordance with the findings of this study. In the study by Pfitzer *et al*. (2011), *A. marginale, Anaplasma (Ehrlichia)* sp. Omatjenne, and *A. bovis* DNA were detected in nyala from four game ranches in the Pongola area, northern KwaZulu-Natal. *Anaplasma centrale* could not be detected in any of the nyala. Most farms on which the nyala were captured had been used for cattle ranching before or share a boundary with cattle-grazing areas.

The lack of a simple *in vitro* bacteriological culture system, the absence of a suitable laboratory animal model, and the inability to distinguish *Anaplasma* species microscopically, all contribute towards the difficulty of classical taxonomic characterisation of species in this group (Lew, *et al.*, 2002). PCR assays based on the 16S rRNA gene have been invaluable for the detection of pathogenic bacteria that are difficult to isolate and grow in the laboratory, and the 16S rRNA gene sequences determined from the amplicons have contributed greatly to phylogenetic studies on eubacteria (Woese, 1987; Dumler, *et al*., 2001). Small-subunit rRNA molecules are structurally and functionally conserved and, therefore, provide a useful starting-point for the phylogenetic classification of organisms. However, one can unfortunately not rely upon 16S gene differences as the sole means for defining genospecies. In this study, 64 samples were selected that either tested (i) positive for a specific *Anaplasma* spp. (*A. centrale, A. marginale* and/or *Anaplasma* (*Ehrlichia*) sp. Omatjenne) using the RLB assay, or (ii) in which the PCR products hybridized only with the *Anaplasma/Ehrlichia* genus-specific probes for molecular characterization by cloning and sequencing of the 16S rRNA gene. We were unable to amplify the full-length and/or partial parasite 16S rRNA gene of any of the selected samples that previously tested positive for the presence of *Anaplasma* (*Ehrlichia*) sp. Omatjenne (using the RLB assay) or *E. ruminantium* (using the pCS20 real-time PCR assay). This was most probably due to low parasitaemia.

The amplification of either the near full-length parasite 16S rRNA gene or a partial 16S rRNA gene from seven samples from the KNP and three from Hluhluwe-iMfolozi Park was successful. BLASTn homology searches indicated that the obtained sequences of 12 of the 18 clones were most similar to published *A. centrale* 16S rRNA gene sequences (accession numbers EF520689; AF318944; AF414869; and EF520688). These were previously identified from cattle in Italy (EF520689, EF520688), cattle from South Africa (AF318944) and from *Rhipicephalus simus* ticks in South Africa (AF414869). Four of the clones were

most similar to the published *A. marginale* sequences (accession no's AF311303, DQ341369, DQ341370 and HM538192) which was previously identified from water buffalo in south China. The sequences of the remaining two clones were closely similar to *Anaplasma* sp. strain Omatjenne (previously *Ehrlichia* sp. strain Omatjenne) (ESU54806) (previously identified from goats and *Hyalomma truncatum* ticks in Namibia) and *Anaplasma platys* (AF399917 and EF139459) (previously identified from dogs in Venezuela and Thailand, respectively). These two agents have not been described previously as pathogens in cattle.

The observed sequence similarities were confirmed by phylogenetic analyses. The phylogenetic trees inferred from both the partial and near-full length 16S rRNA gene clustered into six groups representing *A. centrale*, *A. marginale*, *A. ovis*, *A. phagocytophilum*, *Anaplasma* (*Ehrlichia*) sp. strain Omatjenne / *A. platys* and *E. ruminantium.* This was in concordance with the phylogenetic trees published by Dumler *et al*. (2001) which were based on 16S rRNA and groESL (the gene coding the thermal shock protein) sequences. This systematical division, emerging from the relationship analyses, was also confirmed by the biological and antigenic characterization of each species (Dumler, *et al*., 2001). Lew *et al*. (2002) supported these groupings in 2003 by analysing multiple strains of *A. marginale, A. centrale* and *A. ovis* using 16S rDNA and the GroEL (HSP60) sequence data in an attempt to better differentiate or classify the species within this group. Although the groupings were confirmed, the authors stated that the 16S rDNA analysis could not delineate *A. ovis* isolates from either *A. marginale* or *A. centrale*, as demonstrated by the branching of the phylogenetic tree. This was also evident in the phylogenetic trees generated in our study. Lew *et al*. (2002) further stated that 16S rDNA analyses were useful for assisting to define genera, but that the GroEL sequences were more useful for defining species consistent with phenotypic and traditional taxonomies.

This study has identified *A. centrale, A. marginale* and *Anaplasma (Ehrlichia)* sp. Omatjenne 16S rRNA gene variants. The *A. centrale* sequence variants obtained differed from each other with one to five nucleotides over a 1305 bp region. One of the sequences obtained (HIP/A8/c) was 100% identical to published *A. centrale* 16S rDNA sequences (AF309869, AF318944; AF414869; EF520686, EF520687, EF520688, EF520689 and EF520690). *Anaplasma centrale* AF318944 originated from the South African (Onderstepoort) *A. centrale* vaccine strain (Potgieter, 1979; Bekker, *et al*., 2002), while *A. centrale* AF309869 originated from the

Israel vaccine strain (direct GenBank submission). The *A. centrale* AF414869 sequence was previously identified from *Rhipicephalus simus* ticks in South Africa (Lew, *et al*., 2002), while EF520686, EF520687, EF520688, EF520689 and EF520690 were identified from cattle in Italy (Ceci, *et al*., 2008). The latter being the first report of bovine anaplasmosis by *A. centrale* in Europe. Furthermore, sequence HIP/A8/c differed by only one nucleotide from the Australian *A. centrale* vaccine strain (AF414868). *Anaplasma centrale* is currently the most widely used live vaccine strain for control of bovine anaplasmosis and it is used in several parts of the world, including Africa, Australia, Israel and Latin America (Aubry & Geale, 2011; Kocan, *et al.,* 2000). The occurrence of *A. centrale* DNA in the South African buffalo population is of great interest. Did *A. centrale* evolve in the African buffalo and is buffalo the natural reservoir host of *A. centrale*? Or, was the *A. centrale* vaccine strain transmitted from vaccinated cattle to buffalo populations at some stage and has it established itself in the buffalo population?

Based on the finding of Oura *et al*. (2011) that *A. centrale* was present in the buffalo population in four national parks in Uganda, and recently from two geographic areas in Botswana (unpublished results), it is tempting to speculate that *A. centrale* has evolved in the African buffalo and that buffalo act as natural reservoir hosts. This could play an important role in the epidemiology of bovine anaplasmosis. The main biological vectors and the only successful transmission reported for the mildly pathogenic *A. centrale* appear to be the multihost, strictly African tick species *Rhipicephalus simus* (Potgieter & Van Rensburg, 1987). *Rhipicephalus decoloratus, Rhipicephalus microplus, Rhipicephalus evertsi evertsi* and *Hyalomma marginatum rufipes* (the tick species capable of transmitting *A. marginale* in South Africa) have not been shown to be possible vectors (Potgieter, 1981; De Waal, 2000). The developmental stages of *A. centrale* in ticks have not been described yet (Shkap, *et al*., 2009). Shkap *et al*. (2009) stated that both the failure to transmit *A. centrale* by tick species that are widely distributed in anaplasmosis-endemic regions and the absence of natural infection, on the one hand, and the high prevalence of *A. marginale* infection in the field, on the other hand, might indicate that the transmission capability of *A. centrale* is extremely limited, if indeed it exists. Also, since its first isolation and subsequent re-isolation in South Africa (Theiler, 1911; Potgieter, 1979), no natural *A. centrale* infection in cattle has been reported (Shkap, *et al*., 2009). Cattle become persistently infected carriers after vaccination with live *A. centrale*; infection is subclinical and self-limiting. It is well documented that

cattle that are persistently infected with *A. marginale* are clinically healthy and serve as reservoirs of the rickettsia, irrespective of the pathogen level in the host. However, whether *A. centrale* vaccinates serve as reservoirs for tick transmission is not yet clear (Shkap, *et al*., 2009). This carries important epidemiological implications for countries where anaplasmosis is an economically important disease and where vaccination with *A. centrale* may or may not be applied (Shkap, *et al*., 2009). It remains to be determined whether *A. centrale* has evolved in the African buffalo, does buffalo act as natural reservoir hosts, or is it merely being maintained in the buffalo population by *in utero* transmission?

Although *Anaplasma marginale* causes clinical disease predominantly in cattle, selected ruminants may serve as reservoirs of infection. In this study four *A. marginale* 16S rDNA variants were identified from buffalo from the KNP. These were unfortunately only based on partial 16S rDNA sequence data. The four *A. marginale* variants differed only by two nucleotides from *A. marginale* AF311303 (identified in Virginia, USA) (direct GenBank submission) and DQ341369, DQ341370 and HM538192 identified from water buffalo from south China (direct GenBank submission). However, it was in concordance with the findings of other authors. Several strains of *A. marginale* have been identified from various geographical areas. They differ in morphology, protein sequence, antigenic characteristics and their ability to be transmitted by ticks (Smith, *et al*., 1986; Wickwire, *et al*., 1987; Allred, *et al*., 1990; Rodrigues, *et al*., 2000; De La Fuente, *et al*., 2001, 2003; Palmer, *et al*., 2001). Lew *et al*. (2002) demonstrated a remarkably high conservation of 16S rDNA (and GroEL) sequences among the worldwide collection of strains of *A. marginale* included in their study. They speculated that it may be due to the worldwide dissemination of cattle consequently distributing common ancestors of *A. marginale*. Phylogenetic and phylogeographic studies have also provided evidence that there appears to be a mechanism for maintaining the diversity of various strains of *A. marginale* in nature (Kocan, *et al*., 2010). Therefore, the increasing number of geographic strains recognized that vary in genotype, antigenic composition, morphology, and infectivity for ticks most likely has resulted from extensive cattle movement. Also, the genetic diversity of *A. marginale* strains constitutes a major challenge for developing vaccines that can protect animals against these diverse isolates. Wildlife species, and in this case the African buffalo, may be a factor in the epidemiology and spread of bovine anaplasmosis because, as reservoir hosts of *A. marginale*, they could serve as a source of infective blood for mechanical spread by various routes and biological

transmission by ticks (Kocan, *et al.*, 2010). However, factors such as climate, host abundance, tick host diversity, and topography were all shown to impact the epidemiology of *A. marginale* (Estrada-Peña, *et al.*, 2008).

In this study two *Anaplasma (Ehrlichia)* sp. Omatjenne 16S rDNA variants were also identified. The near-full length 16S rDNA sequence HIP/A8/b differed by four nucleotides from *Anaplasma (Ehrlichia)* sp. Omatjenne (U54805) (previously identified from goats and *Hyalomma truncatum* ticks in Namibia) (Du Plessis, *et al.,* 1990) and by six nucleotides from *A. platys* (M82801) (Anderson, *et al.*, 1992) (type species). This would suggest that sequence HIP/A8/b represents a novel *Anaplasma (Ehrlichia)* sp. Omatjenne sequence. However, when only taking the partial 16S rDNA sequence into account, both HIP/A8/b and HIP/B17/a were identical to *A. platys* EU439943, EF139459, AF399917 which would have suggested that both these obtained sequences represented *A. platys*. *Anaplasma platys* was first detected in the USA in 1978 (Harvey, *et al.*, 1978); it is mainly a pathogen of canines, usually dogs, causing canine cyclic thrombocytopenia (Rymaszewska & Grenda, 2008). A tick vector is suspected, but has not been established. The complete 16S rDNA sequence of *A. platys* is 99.5% identical to that of *Anaplasma (Ehrlichia)* sp. Omatjenne (Allsopp, *et al.*, 1997). This highlights the importance of using full-length sequences if the 16S rRNA gene is to be used for sequence comparison. Moreover, the close identities of the *Anaplasma* species detected in the present study make it difficult to come up with stronger assumptions about species diversity, if only the 16S rRNA gene is used as the basis. The 16S rRNA gene is less variable compared to other genes, because it shares higher similarities among closely related species (Mollet, *et al.*, 1997; Ybañez, *et al.*, 2012).

Anaplasma (Ehrlichia) sp. Omatjenne (an *Ehrlichia*-like agent) was isolated from a *Hyalomma truncatum* tick in a heartwater-free (and *Amblyomma*-free) area of Namibia (Du Plessis, *et al.,* 1990; Allsopp, *et al.*, 1997). It was shown that after several passages of this "*Ehrlichia*-like" agent through *Amblyomma* ticks, sheep developed severe signs of disease similar to heartwater. Northern KwaZulu-Natal as well as regions of Eastern Cape fall within the distribution range of *Hyalomma truncatum* (Walker, *et al.*, 2003), which could explain the occurrence of this species. *Anaplasma (Ehrlichia)* sp. Omatjenne was previously detected in nyala from four game ranches in the Pongola area, northern KwaZulu-Natal (Pfitzer, *et al.*,

(2011), but has not been identified in buffalo before. The significance of this finding remains unclear.

CHAPTER 6

CONCLUSIONS

The occurrence of tick-borne haemoprotozoan parasites in buffalo from two geographically isolated national parks in South Africa (Kruger National Park and Hluhluwe-iMfolozi Park) was determined. Results revealed the presence of both pathogenic and non-pathogenic *Theileria* species, *Babesia occultans, Anaplasma marginale, A. centrale*, *Anaplasma* (*Ehrlichia*) sp. Omatjenne and *E. ruminantium*. Previous studies on the occurrence of haemoparasites in the South African buffalo population have mainly focussed on the prevalence of *Theileria* species only. The finding on the presence of *Anaplasma, Ehrlichia* and *Babesia* species is therefore a novel contribution.

This study has confirmed the findings of previous studies that buffalo are commonly co-infected with different *Theileria* spp., namely, *T. parva*, *Theileria* sp. (buffalo), *T. mutans*, *T. velifera* and *T. buffeli*. In addition, *Theileria* sp. (sable) DNA was also detected in the buffalo population in KNP and Hluhluwe-iMfolozi Park. *Babesia occultans*, the causative agent of a benign form of cattle babesiosis in South Africa, was detected in 50% of the buffalo from the Hluhluwe-iMfolozi Park. To date, this organism had not been identified in wildlife in South Africa. The significance of detecting *B. occultans* DNA in buffalo from the Hluhluwe-iMfolozi Park is unclear and should be confirmed using cloning, sequencing and phylogenetic analysis. The possible risk thereof to antelope species also warrants further investigation.

Although *A. marginale* and *E. ruminantium* have been reported to infect wild ruminants and have been detected in wildlife, little is known about the susceptibility of buffalo to *A. marginale* and/or *E. ruminantium* infection or the role of buffalo in the epidemiology of the diseases caused. *Ehrlichia ruminantium* DNA was detected in 5% of the KNP samples investigated using the quantitative pCS20 real-time PCR assay (Steyn, *et al*., 2008). The significance of this finding remains unknown; these results would suggested that buffalo is

not the natural reservoir host of *E. ruminantium*. However, a subclinical carrier state in buffalo has been experimentally shown to occur after tick transmission from carrier animals and further studies will have to be done to confirm whether this finding holds any potential risk to domestic animals.

Anaplasma marginale, *A. centrale* and *Anaplasma (Ehrlichia)* sp. Omatjenne DNA was detected in the buffalo from both the KNP and Hluhluwe-iMfolozi Park. Some of these samples were selected for molecular characterization by cloning and sequencing of the 16S rRNA gene. Results indicated the presence of *A. marginale*, *A. centrale* and *Anaplasma (Ehrlichia)* sp. Omatjenne 16S rDNA variants. An interesting finding was the presence of one 16S rDNA sequence that was 100% identical to that of the published *A. centrale* vaccine strain 16S rDNA sequences. It is well known that *A. centrale* is widely used as live vaccine for the control of bovine anaplasmosis. The occurrence of *A. centrale* vaccine strain DNA in the South African buffalo population is therefore of great importance as it could impact on the use of *A. centrale* live vaccines. It can only be speculated on whether *A. centrale* has evolved in the African buffalo, and/or if buffalo act as natural reservoir hosts, or if is it merely being maintained in the buffalo population by *in utero* transmission.

The presence of *A. marginale* in the buffalo population suggests that buffalo may be a factor in the epidemiology and spread of bovine anaplasmosis because, as reservoir hosts of *A. marginale*, they could serve as a source of infective blood for mechanical spread by various routes and biological transmission by ticks. Factors such as climate, host abundance, tick host diversity, and topography have, however, all been shown to also impact on the epidemiology of *A. marginale*.

In conclusion, findings of this study suggest that buffalo are a natural reservoir of *Anaplasma* spp. infection and could play an important role in the epidemiology and spread of anaplasmosis and may represent a serious threat to the livestock industry. Further studies should also be done to include the screening of buffalo and other wildlife species from neighboring reserves as well as other national park throughout the country for the presence of *Anaplasma* species. The knowledge derived from such studies would contribute significantly

to the epidemiological map of these pathogens and would aid authorities in disease management and control.

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