

**Tick-borne haemoparasite prevalence and *Theileria parva* strain diversity
in African buffalo (*Syncerus caffer*) from northern Botswana and
Gonarezhou National Park, Zimbabwe**

by

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DEDICATION

Dedicated to my mother, Agnes Eygelaar and my girlfriend, Crystal du Toit, the people who were always there for me throughout this project even though they not always understand what I was ranting about. I appreciate both your love for me and confidence in me.

Dankie Ou vrou

Dankie Crys

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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25 November 2015

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ABBREVIATIONS

°C: degrees Celsius
A: Adenine
bp: base pair
BLAST: basic local alignment search tool
C: Cytosine
CD4⁺: cluster of differentiation 4 glycoprotein
CD8⁺: cluster of differentiation 8 glycoprotein
CNP: Chobe National Park
DNA: deoxyribonucleic acid
dNTP: deoxynucleotide triphosphates
DVTD: Department of Veterinary Tropical Diseases
ECF: East Coast fever
EDTA: Ethylenediaminetetraacetic acid
ELISA: Enzyme-linked immunosorbent assay
Flt3L: fetal liver tyrosine kinase 3 ligand growth factor
FTA: Flinders technology associates
G: Guanine
GNP: Gonarezhou National Park
h: hour
IFAT: indirect fluorescent antibody test
IFN- γ : interferon-gamma
kDa: kilodalton
KNP: Kruger National Park
 μ l: microliter
mg: milligram
ml: millilitre
mM: millimolar
min: minute
ng: nanogram
NS1: Non-structural protein 1
OD: Okavango Delta
OVI: Onderstepoort Veterinary Institute
p104: *Theileria parva* 104 KDa antigen gene
p67: *Theileria parva* sporozoite antigen gene

PIM: polymorphic immunodominant molecule

pmol: picomoles

PCR: polymerase chain reaction

RFLP: restriction fragment length polymorphism

qPCR: quantitative real time PCR

RLB: reverse line blot

rRNA: ribosomal ribonucleic acid

SDS: sodium dodecyl sulphate

sp.: species

SSPE: standard saline phosphate/EDTA

T: Thymine

TBDs: tick-borne diseases

TE: Tris-EDTA

U: enzyme units

**TICK-BORNE HAEMOPARASITE PREVALENCE AND *THEILERIA PARVA* STRAIN
DIVERSITY IN AFRICAN BUFFALO (*SYNCERUS CAFFER*) FROM NORTHERN
BOTSWANA AND GONAREZHOU NATIONAL PARK, ZIMBABWE**

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SUMMARY

The African buffalo (*Syncerus caffer*) is host for many pathogens known to cause economically important diseases and is often considered an important reservoir for livestock diseases. Theileriosis, heartwater, babesiosis and anaplasmosis are considered the most important tick-borne diseases of livestock in sub-Saharan Africa, resulting in extensive economic losses to livestock farmers in endemic areas. In this study a variety of tick-borne haemoparasites (*Theileria*, *Babesia*, *Anaplasma* and *Ehrlichia* species) were identified either as mixed or single infections using the reverse line blot (RLB) hybridization assay from buffalo blood samples in the Chobe National Park (CNP) and Okavango Delta (OD), Botswana and in the Gonarezhou National Park (GNP), Zimbabwe. Also, a quantitative real-time PCR (qPCR) assay was used to identify *Theileria parva* more specifically in both these countries while the indirect fluorescent antibody test (IFAT) was used to identify *Theileria parva* more specifically in Botswana only. An attempt was made to characterize *T. parva* through the size differentiation of p67 genotypes and characterization of the variable regions of *T. parva* antigen genes, p104 and PIM, by using semi-nested PCR-RFLP profiles. This is the first report of tick-borne haemoparasites in northern Botswana and one of only a few from Zimbabwe.

This study identified the following tick-borne haemoparasites: *Theileria* spp. present, *T. parva* (60%) and *T. mutans* (37%) were the most prevalent in the two wildlife areas from Botswana, while *Theileria* sp. (sable) (50%), *T. parva* (48%) and *T. mutans* (38%) were most prevalent in GNP, Zimbabwe. Other species of interest were *Anaplasma marginale* subsp. *centrale* (30%), *A. marginale* (20%), *Babesia occultans* (23%) and *Ehrlichia ruminantium* (6%) in Botswana and *Anaplasma marginale* subsp. *centrale* (25%) and *Babesia occultans* (15%) in Zimbabwe. Generally speaking, the buffalo population in the OD sample had lower levels of haemoparasite infection than the buffalo in the CNP and GNP, with the exception of *Theileria* sp. (buffalo) and to a lesser extent *Anaplasma* sp. Omatjenne and *B. bovis* (in the two later cases, where very few positives were detected).

Interesting findings included: *Anaplasma* sp. Omatjenne identified in this study, another research group identified 16.5% to be positive in their samples, but the parasite was found in very low concentrations (3.1%) in our study. *B. occultans* causes a benign form of cattle babesiosis and was also reported in South Africa by this research group for the first time. Our study identified 21.1% samples to be positive compared to the study in Hluhluwe-iMfolozi Park, South Africa (50.0%). This study serves as another report of the presence of these two parasites in buffalo. As in Uganda, the pathogenic *B. bovis* has previously been reported to be absent from buffalo in Botswana but were identified at a low concentration in OD. Similarly, *E. ruminantium* could be identified in a few CNP and OD buffalo tested. The significance of buffalo as possible reservoir host of some of these economically important haemoparasites (i.e. *A. marginale*, *E. ruminantium*) remains unknown. *Theileria* sp. (sable), which is fatal to sable (*Hippotragus niger*) and roan antelope (*Hippotragus equinus*), but non-pathogenic to buffalo was identified in some of the Botswana and Zimbabwe buffalo but positive RLB signals might be due to cross reactions of the *Theileria* sp. (sable) probe with *T. velifera*.

Theileriosis is recognized as a major threat to the livestock industry as some members of the genus may cause severe disease and mortality, whereas others may only cause mild or subclinical infections. In this study the efficiency of IFAT, qPCR and RLB in identifying *T. parva* were compared to each other. qPCR was the most effective (81%) followed by IFAT (74%) and then RLB (60%) in Botswana. In Zimbabwe, qPCR (70%) identified more samples to be positive than RLB (48%). The level of agreement between the tests for detection of *T. parva* positive animals was higher between qPCR and IFAT (kappa=0.56), than between qPCR and RLB (kappa=0.26) or the latter and IFAT (kappa=0.15) in Botswana. The kappa agreement between qPCR and RLB in Zimbabwe was 0.27. The RLB, IFAT and qPCR tests all indicated a high prevalence of *T. parva* in the study areas. This indicates a high risk of spreading Corridor disease caused by *T. parva* from buffalo to cattle by the vector ticks at the wildlife-livestock interface.

Several *T. parva* antigen genes have been identified as good candidates for differentiation between buffalo-derived and cattle-derived *T. parva* isolates. Some of these genes include: p67, p104 and the polymorphic immunodominant molecule (PIM). These genes were amplified in an attempt to differentiate between buffalo-derived and cattle-derived profiles. Amplification of p67 in this study led to the identification of three of the four known p67 alleles. Cluster analysis for p104 showed that samples from Botswana and Zimbabwe clustered together in clade B with themselves and with samples from Hluhluwe while all samples from the Kruger National Park clustered in clade A with samples from Ladysmith. The cluster analysis of PIM revealed that samples from this project (Botswana and Zimbabwe) cluster in four clades, distinct from all samples from South Africa, which, except for one sample from Hluhluwe, clustered in a single clade.

In conclusion, this study highlights the diversity of haemoparasites present in African buffalo from northern Botswana and Zimbabwe and also the role of African buffalo as a sentinel species for livestock tick-borne pathogens. Important tick-borne haemoparasites identified in this study included: *T. parva*, *A. marginale*, *B. bovis* and *E. ruminantium*. This study reconfirmed that p67 profiles are too complex and could not be used to distinguish between cattle- and buffalo-derived *T. parva* isolates. Mixed infections of p104 and PIM profiles generated by PCR-RFLP analysis were too complex to successfully differentiate between known profiles. Further cloning and sequencing of single infections are needed.

Chapter 1

General Introduction

Theileriosis is a widespread disease of wild and domestic ruminants caused by tick-transmitted apicomplexan parasites of the genus *Theileria*. Theileriosis is recognized as a major threat to the livestock industry as some members of the genus may cause severe disease and mortality, whereas others may only cause mild or subclinical infections. By far the most pathogenic and economically significant *Theileria* sp. in eastern, central and southern Africa is *T. parva*, which appears to have co-evolved in the African buffalo (*Syncerus caffer*) (Norval et al. 1992), and causes East Coast fever (ECF), Corridor disease and January disease in cattle (Uilenberg et al. 1982; Perry et al. 1991). Whilst causing only subclinical infection in buffalo, *T. parva* causes fatal disease in cattle. *T. parva* is transmitted by the three-host ticks, *Rhipicephalus appendiculatus*, *Rhipicephalus zambeziensis* and *Rhipicephalus duttoni* (Lawrence et al. 1983; Norval et al. 1992).

Theileria parva was historically classified into three sub-species, namely *T. parva parva* (causative agent of ECF), *T. parva bovis* (causative agent of January disease) and *T. parva lawrencei* (causative agent of Corridor disease). However, this classification was abandoned due to a lack of biological evidence as these sub-species are morphologically and serologically indistinguishable (Norval et al. 1992; Allsopp et al. 1993), and *T. parva* is now the only recognised species. It has since been recommended that parasites that circulate in cattle and cause ECF and January disease are referred to as cattle-derived *T. parva* and parasites that originate from buffalo, which cause Corridor disease, are referred to as buffalo-derived *T. parva* (Perry and Young 1993).

Several assays have been used to attempt to differentiate between cattle- and buffalo-derived *T. parva* stocks, none being 100% successful (Minami et al. 1983; Allsopp et al. 1989; Conrad et al. 1989; Bishop et al. 1993; Collins and Allsopp 1999). Size polymorphism displayed by *T. parva* antigen genes has been used to develop several molecular tools for characterization of *T. parva* stocks, including the sporozoite antigen gene p67, the polymorphic immunodominant molecule (PIM) and p104 (Iams et al. 1990; Nene et al. 1996; Geysen et al. 1999; Bishop et al. 2001). In recent studies by Sibeko and colleagues (Sibeko et al. 2010, Sibeko et al. 2011) it was evident that *T. parva* parasites that exist in South African buffalo and cattle were genetically far more diverse than those found in cattle in East Africa. It was shown that PIM and p104 polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) profiles are more complex than previously thought and, therefore, the classification of buffalo-derived and cattle-derived *T. parva* parasites in South Africa based on these profiles was not possible. Size differentiation and sequence variation of the central region of the

T. parva p67 gene could also not be used successfully to differentiate between cattle- and buffalo-derived *T. parva* parasites although the study revealed the presence of p67 genotypes also found in *T. parva* isolates from East and South Africa.

In this study, buffalo were sampled from northern Botswana (Chobe National Park and the Okavango Delta) and Zimbabwe (Gonarezhou National Park) and screened for the presence of *T. parva* using the *T. parva*-specific 18S rRNA real-time PCR assay (Sibeko et al. 2008). Since no molecular data is currently available on the antigen genes of *T. parva* present in buffalo from Botswana and little from Zimbabwe (Bishop et al. 1994), *T. parva* parasites were characterized using PCR-RFLP profiles of the p67, PIM and p104 genes also in an attempt to classify them as buffalo- or cattle-derived.

The specific objectives were:

- (i) Screening of buffalo blood samples originating from Botswana and Zimbabwe for the presence of *Theileria*, *Babesia*, *Anaplasma* and *Ehrlichia* spp. using the Reverse Line Blot (RLB) hybridization assay.
- (ii) Screening for the presence of *T. parva* using the *T. parva* specific real-time PCR assay.
- (iii) Size differentiation of the central region of the p67 *T. parva* antigen gene.
- (iv) Characterization of the variable regions of the *T. parva* antigenic genes, PIM and p104, using semi-nested PCR-RFLP profiles.
- (v) Comparison of the *T. parva* p104 and PIM antigenic genes PCR-RFLP profiles obtained with those available from South Africa.

Chapter 2

Literature Review

Theileriosis is considered to be one of the most important tick-borne diseases of livestock in sub-Saharan Africa, resulting in extensive economic losses to farmers in endemic areas. Corridor disease, caused by *Theileria parva*, is a controlled disease in South Africa. The African buffalo is the natural reservoir host of this parasite, which is transmitted by *Rhipicephalus appendiculatus*, *R. zambeziensis* and *R. duttoni* (Norval et al., 1991; Uilenberg, 1999). Buffalo also appear to be the original hosts of two other *Theileria* species infecting cattle, the relatively benign *T. mutans* and the apathogenic *T. velifera*. *Theileria buffeli*, *Theileria* sp. (buffalo) and *Theileria* sp. (bougasvlei) have been described in the African buffalo. *Theileria* parasites usually occur as mixed infections and the benign and non-pathogenic forms do not have any significant economic importance. However, their presence could interfere with the interpretation of results obtained in some diagnostic tests designed to diagnose the pathogenic *T. parva* (Chaisi et al. 2013). Risk factors influencing the prevalence of tick-borne parasites may include the distribution of tick vectors, the abundance of buffalo and cattle and their movement/migratory patterns, resistance of the hosts to the parasites and their tick vectors (Bakheit and Latif, 2002) and age of the host (Anderson et al. 2013). Indeed, it has previously been found that older animals have a higher tick load than younger ones (Anderson et al. 2013). In addition to *Theileria*, it has been described that African buffalo are also carriers of a number of other tick-borne parasites which are seriously detrimental to livestock health and production, including *Ehrlichia ruminantium*, *Babesia bigemina*, *B. bovis*, *Anaplasma marginale* and *A. marginale* subsp. *centrale* (Andrew and Norval, 1989; Iseki et al., 2007; Kuttler, 1965).

2.1. Classification of *Theileria* spp.

The super-group Chromalveolata contains the phylum Apicomplexa, which *Theileria* forms part of (Adl et al., 2005). *Theileria* parasites are single celled, apicomplexan eukaryotes that contain an apical complex. Both schizont and piroplasm stages are present in mammalian cells; in the lymphoblasts and erythrocytes, respectively. The sporozoite stage can be found in the tick vector (Irvin, 1983).

Classification (Adl et al. 2005):

Supergroup: Chromalveolata
Superphylum: Alveolata
Phylum: Apicomplexa
Class: Aconoidasida

Order: Piroplasmida

Family: Theileriidae

Genus: *Theileria*

2.2. Life cycle of the *Theileria* parasite in the mammalian host and the tick vector:

Theileria species have complex life cycles that involve several morphologically distinct developmental stages in tick and mammalian host cells (Fig. 1). The transmission and the survival of the parasite depend on the ability of various invasive stages (the sporozoite and merozoite in the mammalian host, the zygote and kinete in the tick vector) to recognize and invade specific host cells (Shaw, 2003).

The life cycle of *T. parva* starts in the form of sporozoites that develop in the salivary glands of ticks where they mature to full size (Stagg *et al.* 1980). When the tick feeds, the sporozoites infiltrate the host's lymphocytes where they differentiate to form schizonts (Stagg *et al.* 1980). These schizonts cause transformation of infected lymphocytes which leads to the clonal expansion of schizonts (lymphoblasts). In the lymphocytes, schizonts differentiate to form merozoites at a later stage in the cycle. Merozoites have the ability to invade red blood cells, in which they develop into piroplasms. Piroplasms are taken with blood when a new tick (nymph) feeds and the life cycle continues in the gut of the tick. Here, gametogenesis and fertilization occur to produce a zygote (Melhom and Schein 1984). The zygote then enters the gut cells, where it transforms into a single motile kinete. Kinetes travel to the salivary gland of the adult tick where they stay dormant until the tick feeds again, upon which sporozoites are rapidly formed in the gland. In future when the adult tick feeds again the sporozoites infect the new host and the cycle starts all over again (Norval *et al.* 1992).

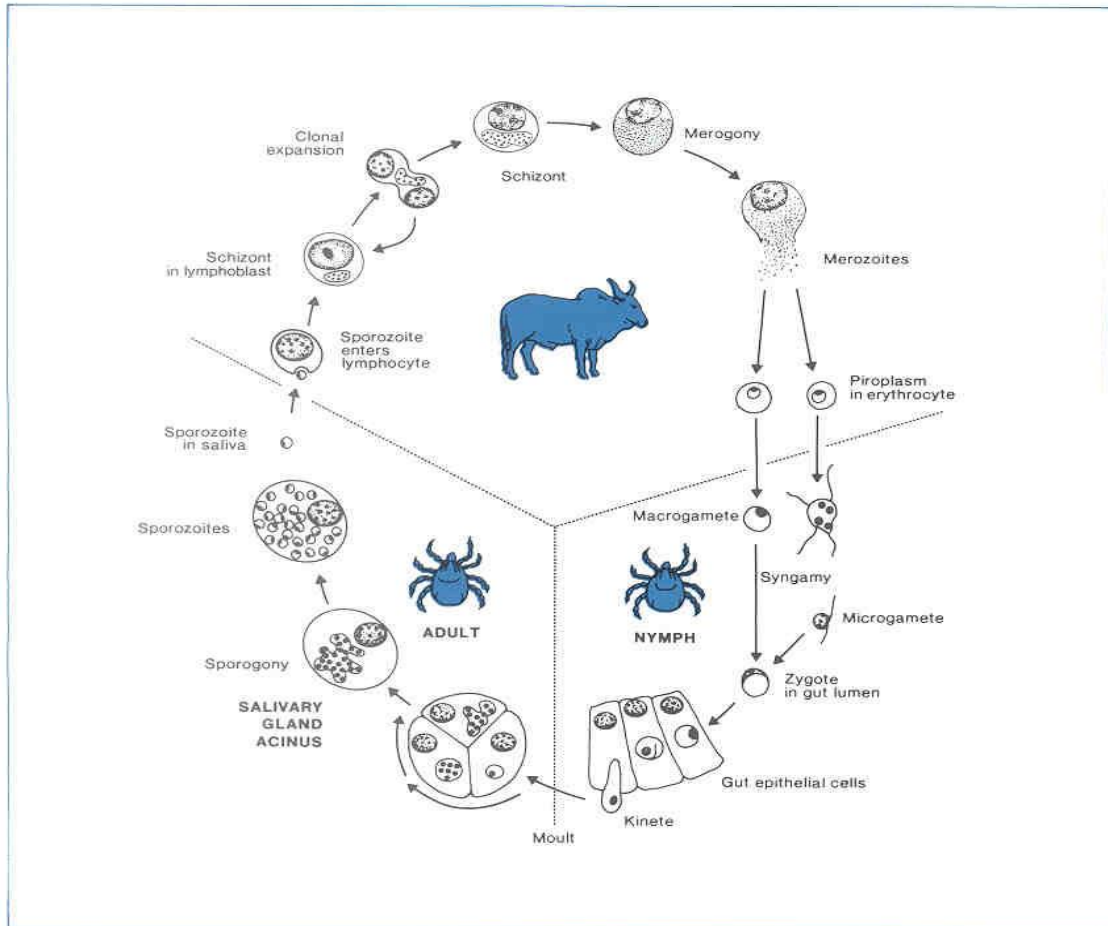


Figure 1: Life cycle of *Theileria parva* (from www.ilri.org/InfoServ/Webpub/fulldocs/Irad90/Theile.htm)

2.3. Benign, mildly pathogenic and severely pathogenic *Theileria* species of buffalo and cattle in southern Africa

2.3.1. Pathogenic *Theileria* species

The most important economic *Theileria* species are *T. parva* and *Theileria annulata*. These are the species that cause significant losses to the cattle industry because of high morbidity and mortality and the costs of treating and controlling these parasites (Allsopp et al. 1993; Schnittger et al. 2002).

Theileria parva has been identified in buffalo and cattle from central, eastern and southern Africa and causes East Coast fever (ECF), Corridor disease and January disease in cattle (ILRAD, 1990). *T. parva* is transmitted by the three-host ticks, *Rhipicephalus appendiculatus*, *Rhipicephalus zambeziensis* and *Rhipicephalus duttoni* (Lawrence et al. 1983; Norval et al. 1992). The African buffalo (*Syncerus caffer*) is the natural reservoir host of *T. parva* (Barnett and Brocklesby, 1966). The waterbuck (*Kobus defassa*) has been experimentally infected with *T. parva* and is thus thought to be a

possible reservoir host (Stagg et al. 1994). *Theileria annulata* causes tropical theileriosis which has high levels of mortalities in exotic cattle breeds and low levels in indigenous breeds (Neitz, 1957). It has been identified in camels, cattle, water buffalo and yak in Central Asia, the Middle East, northern Africa and southern Europe (Sergent et al. 1935). The vectors for this parasite include ticks from the *Hyalomma* genus (Uilenberg, 1981).

2.3.2. Benign and mildly pathogenic *Theileria* species

Theileria mutans which is transmitted by *Amblyomma* ticks was identified in buffalo and has been found to infect cattle (Paling et al. 1981; Uilenberg et al. 1982; Allsopp et al. 1993). The parasite is considered to be benign in buffalo but has been reported to cause disease in cattle (Young et al. 1978; Paling et al. 1981).

Theileria sp. (buffalo) was first identified in buffalo in Kenya, East Africa (Conrad et al. 1987a) and it has subsequently been found in buffalo from Uganda (Oura et al. 2011) and South Africa (Chaisi et al. 2011; Pienaar et al., 2011) but its vectors remain unknown (Norval et al. 1992).

Theileria sp. (bougasvlei), a species closely related to *Theileria* sp. (buffalo) and *T. parva* has been identified in buffalo and reported previously (Zweygarth et al., 2009). Speciation of *Theileria* sp. (buffalo) and *Theileria* sp. (bougasvlei) has been difficult because of their close similarities. Work done by Mans et al. (2011) compared the rRNA V4 hyper-variable of *T. parva*, *Theileria* sp. (buffalo) and *Theileria* sp. (bougasvlei) and found only 3-5 nucleotide differences between them. Morphologically there is no difference between *Theileria* sp. (buffalo) and *Theileria* sp. (bougasvlei) but the latter has lower parasitemia levels while *Theileria* sp. (buffalo) has higher levels than *T. parva* (Zweygarth et al., 2009; Pienaar et al., 2013).

Theileria buffeli/sergenti/orientalis causes a benign infection in buffalo and cattle from Africa, Asia, Australia, Europe and the United States of America (Chae et al. 1998; Chansiri et al. 1999; Cossio-Bayugar et al. 2002; Aktas et al. 2007; Altay et al. 2008; M'ghirbi et al. 2008; Gimenez et al. 2009; Chaisi et al. 2011; Mans et al. 2011). These parasites are transmitted by *Haemaphysalis* ticks in Asia, Australia and Europe but the vectors in the other countries remain unknown (M'ghirbi et al. 2008). *Theileria buffeli* was first identified in Asian water buffalo (*Bubalus bubalis*) in 1908 by Schein (reviewed by Fujisaki et al. 1994), *T. orientalis* and *T. sergenti* were identified in eastern Siberia in the early 1930s (Yakimov and Dekhterev, 1930; Yakimov and Sudachenkov, 1931). Some confusion still remains whether these three parasites were wrongly classified and in fact represent a single parasite but differences in the morphology of macroschizonts and piroplasms have been noted (Uilenberg et al. 1985). Traditionally, the name, *T. sergenti* was used for the species but this actually refers to a parasite of sheep and is currently used incorrectly (Uilenberg, 2011). Gubbels (2002)

pointed out that the species name *T. orientalis*, implies that the parasite is only present in the Far East which is not true because the parasite is found all around the world, and therefore suggested that members of the *Theileria buffeli/sergenti/orientalis* group should be referred to as *T. buffeli* until better classification is available. A study has shown that *T. buffeli* is more diverse than previously thought and a cosmopolitan species (Chaisi et al. 2014). Another species, *Theileria sinensis* which is closely related to *Theileria buffeli/sergenti/orientalis* has also been identified and causes bovine theileriosis in cattle and yak in China (Bai et al. 2002a; Bai et al 2002b, cited by Yin et al., 2004). *T. sinensis* and *T. sergenti* are indistinguishable from each other (Yin et al. 2002) except for their tick vectors, *Haemaphysalis qinghaiensis* and *Haemaphysalis longicornis* respectively (Liu et al 2010). There are also speculations of the occurrence of *T. sinensis* in African buffalo from South Africa (Chaisi et al. 2014).

Theileria taurotragi was first described in eland (*Taurotragus oryx*) in Kenya but has also been identified in cattle (Martin and Brocklesby, 1960; Uilenberg et al, 1982; Stagg et al. 1983); there have been no reports of infections in African buffalo. The parasite has been found in mixed infections with other *Theileria* species in cattle with the main vectors being *R. appendiculatus* and *R. zambeziensis* (Uilenberg et al. 1982; Lawrence et al. 1983). Bovine cerebral theileriosis and Tzaneen disease have been associated with *T. taurotragi* infections in South Africa (De Vos and Roos, 1981; Stoltsz, 1989).

Theileria velifera was first identified in cattle and causes a asymptomatic infection in these animals as well as in African buffalo (Uilenberg, 1964; Norval et al. 1992; Oura et al. 2005). The parasite is transmitted by ticks from the *Amblyomma* genus (Norval et al. 1992).

2.4. Epidemiology of Theileriosis in southern Africa

2.4.1. East Coast fever

The name East Coast fever was adopted for this disease due to the fact that it was enzootic to the East Coast of Africa. *T. parva* was introduced simultaneously into former southern Rhodesia and South Africa in 1901 by imported infected cattle from East Africa (Lawrence, 1979). ECF was introduced to Mozambique through importation of cattle infested with infected ticks from East Africa through the ports of Beira and Maputo in 1901/2 (Lawrence, 1992). The disease was eradicated in South Africa between 1948 and 1954 and this could only be done through a costly procedure where infected farms were put under quarantine and all the animals on infected farms were culled with compensation to the farmers (Anonymous 1981). Zimbabwe was declared ECF free in 1954 because of strict chemical tick control and control of cattle movement (Latif and Hove, 2011). In 1960, Swaziland was the last southern African country to report the eradication of ECF and since then cattle-derived *T. parva* never

again got established in South Africa (Lawrence et al. 1994a). The disease was later (1960s) eradicated from southern Mozambique (Lawrence, 1992). During an ECF epidemic in the eastern province of Zambia, between 1994 and 2000 three cattle herds were followed clinically and serologically. Results showed that the highest disease incidence occurred in the rainy seasons of December–February and the dry months of May–July. Both time periods coincide with maturation of *Rhipicephalus appendiculatus* to its adult form (Billiouw et al., 2002), the disease still persists today throughout Zambia (Lawrence et al. 1994a). Through the use of immunization of calves these epidemics have been brought under control (Marcotty et al. 2002).

2.4.2. Corridor disease

Corridor disease was first recognized in South Africa in 1953 and was found between the then Hluhluwe and Umfolozi Game Reserves in KwaZulu-Natal Province (KZN). This area between the two game reserves formed a corridor and this is where the name of the disease - Corridor disease - originated (Neitz et al. 1955). Corridor disease occurs throughout eastern and southern Africa where the *R. appendiculatus*, *R. zambeziensis* or *R. duttoni* ticks transmit the disease between buffalo and cattle, where they come into contact with each other (Neitz et al. 1955; Blouin and Stoltsz, 1989). Buffalo act as reservoirs for this parasite and the transmission of the parasite from buffalo to cattle is possible. This disease still persists in South Africa and Zimbabwe (Uilenberg, 1999). Mbizeni et al. (2013) showed that from 2004 there has been an increase in outbreaks every year in South Africa. Work done by Yusufmia et al. (2010) suggests that in South Africa not only buffalo but also cattle are subclinical carriers of *T. parva*. The translocation of buffalo from parks in KZN and the Kruger National Park to other parts of the country is strictly prohibited to prevent a possible spread of the disease as well as foot and mouth disease (FMD) (Meltzer, 1993). Breeding projects to obtain “disease-free” buffalo has allowed limited movement of these buffalo to non-endemic areas, subject to strict screening and permits. In a recent study, Chaisi et al. (2011) identified the circulation of *T. parva* and other *Theileria* species in buffalo from the Limpopo National Park, in Mozambique. *T. parva*-causing Corridor disease has been identified in the southern, central, Lusaka and Copper-belt provinces of Zambia, while ECF was more often identified in the northern and eastern provinces (Makala et al. 2003). Later studies identified ECF causing parasites to also be present in the southern and central provinces of Zambia (Minjauw et al. 1998; Billiouw et al. 2005).

2.5. Different disease syndromes caused by *Theileria parva*

In the past, *T. parva* was divided into three subspecies based on the clinical and epidemiological features of the different diseases they caused, with *T. parva parva* causing ECF, *T. parva bovis* causing January disease and *T. parva lawrencei* causing Corridor disease. It has been recognized that there is no biological evidence to support the separate subspecies (Norval et al. 1992; Allsopp et al. 1993) and currently *T. parva* is the only recognised species and different *T. parva* parasites are

classified according to their host of origin, i.e. cattle-derived or buffalo-derived (Anonymous, 1989). Nevertheless, *T. parva* causes recognisably different disease syndromes. Countries where areas of distribution of cattle and one of the tick vectors (*R. appendiculatus*) overlap are considered areas of concern when looking at the different disease syndromes (Fig. 2).

2.5.1. East Coast fever (ECF)

ECF is caused by cattle-derived *T. parva* which is fatal to cattle and was first recognized in 1902 in Rhodesia (currently known as Zimbabwe) (Gray and Robertson, 1902), but the disease was finally eradicated from this country in 1954 (Latif and Hove, 2011). It is transmitted between cattle through infected ticks. Characteristics initially include swollen lymph nodes near bite marks and fever. Death may occur in 18-30 days after infected ticks attach to the animals; mostly due to lung oedema (Morrison *et al.* 1989). Susceptibility towards the disease varies between cattle, some will perish but others might recover and they will become carriers of the parasite and serve as reservoirs. Animals that recover from the disease show lower amounts of fat reserves and muscle content. ECF can be controlled by the use of acaricides, chemotherapy, immunization, pasture management and by keeping infected animals separated from those that are healthy.

2.5.2. Corridor disease

Corridor disease is clinically very similar to ECF but death in cattle is often fulminant and it has a very low level of parasitemia and lower number of schizonts compared to ECF (Nene *et al.* 1996, Lawrence *et al.* 1994b). The disease is caused by buffalo-derived *T. parva* which is transmitted by ticks from buffalo to cattle when both species share the same environment in which infected ticks can pass from wild to domestic hosts. Outbreaks of the disease occur near where infected buffalo graze (Thompson *et al.* 2008). Buffalo act as carriers and do not develop any clinical symptoms of the disease. However, Corridor disease is so fulminant in cattle that the disease is considered to be self-limiting. Indeed, new infective piroplasms are unable to be produced in sufficient numbers because the animal dies too quickly to be able to infect ticks. In South Africa, infected buffalo that come into contact with cattle is the greatest threat of *T. parva* infection in the country (Thompson *et al.* 2008). Most outbreaks in South Africa are encountered during the rainy season which is also the active period of the *R. appendiculatus* adult tick (Walker *et al.* 2000). The disease may be controlled through the sustained separation of cattle and buffalo herds. Chemotherapy and immunization of animals are not allowed in South Africa, to prevent the appearance of carriers that could allow for the re-emergence of ECF (Potgieter *et al.* 1988; Stoltsz, 1989).

The concern is that East Coast fever might re-emerge through the transformation of buffalo-derived *T. parva* into cattle-derived *T. parva* when there is transmission between buffalo and cattle and then these parasites are subsequently transmitted amongst cattle like in the case of ECF (Barnett and

Brocklesby, 1966). Previously, attempts have been made to transmit *T. parva* by tick vector between cattle to test for the selection of parasites adapted to cattle hosts (Neitz et al. 1955; Mbizeni et al. 2013; Thompson et al. 2008). *T. parva* from infected and recovered cattle have been used to infect susceptible cattle but these attempts to demonstrate transformation have been unsuccessful in South Africa. An explanation for this failure might be that there was a too low parasitaemia levels at the time of tick feeding (Mbizeni et al. 2013).

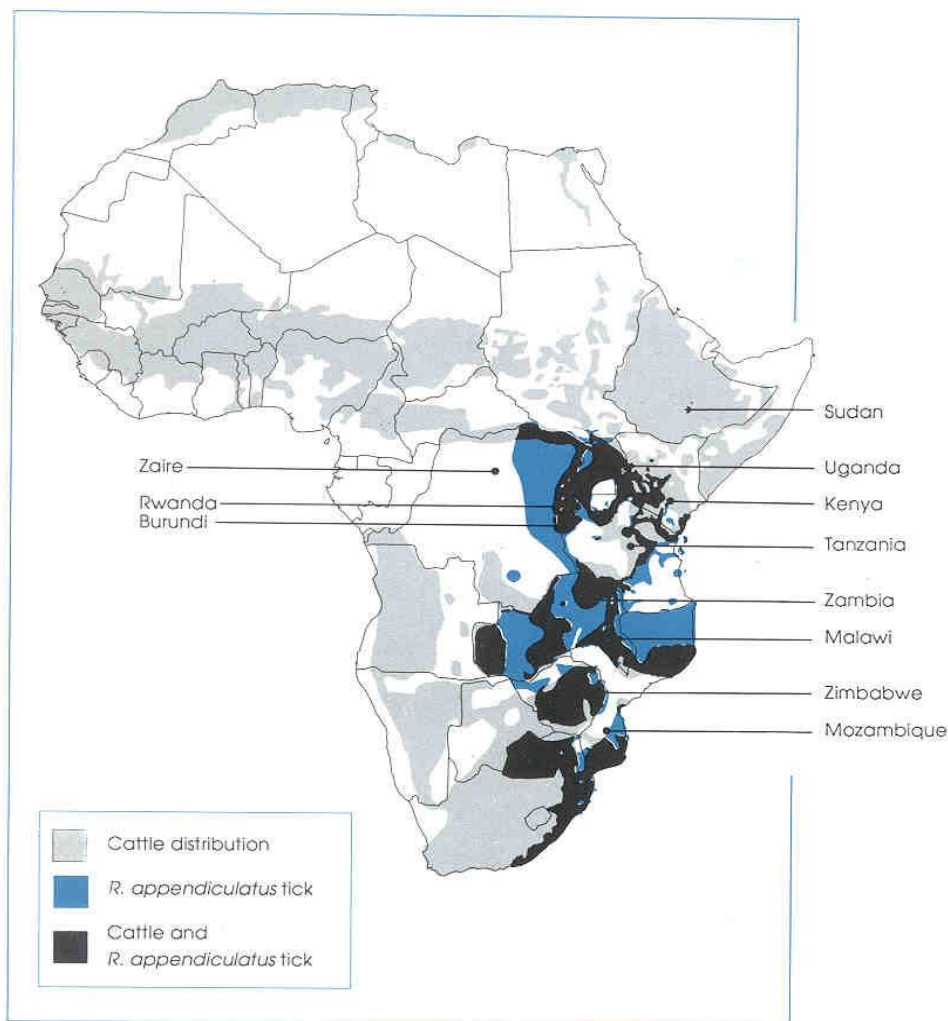


Figure 2: The countries of Africa, showing the distribution of cattle and the tick, *R. appendiculatus*, the vector that transmits *T. parva* to cattle (<http://www.ilri.org/InfoServ/Webpub/fulldocs/Ilrad90/Epidemo.htm>).

2.5.3. January disease (Zimbabwean theileriosis)

This disease is a derivative of ECF caused by *T. parva* (formerly known as *T. parva bovis*) (Lawrence et al. 1994c). The main differences are that this disease is a milder form of ECF and is seasonal, only occurring from December to March, hence the name. January disease. It is transmitted by *R. appendiculatus* and has been experimentally transmitted by *R. zambeziensis* amongst cattle, whose

activity corresponds to the seasonality of the disease. January disease circulates in Zimbabwe and has not been described in other southern African countries (Lawrence et al. 1994c).

2.6. Tick vectors

Rhipicephalus appendiculatus (Brown ear tick) is known to be the main vector of *T. parva* although *R. zambeziensis* and *R. duttoni* have also been shown to be vectors (Uilenberg, 1999). Adult *Rhipicephalus appendiculatus* from the family *Ixodidae* (hard ticks) are usually found on the ears of cattle as the common name implies (Olwoch *et al.* 2008). This tick's main hosts include buffalo, cattle and other large antelopes, but can also be found in small livestock namely sheep and goats. They can be found in the eastern, central and south eastern parts of Africa. *T. parva* is transmitted between hosts through infected ticks. Sporozoites from the salivary glands of adult or nymphal ticks are introduced into the susceptible animal while the ticks feed on the host. According to Norval et al. (1982), *Rhipicephalus zambeziensis* is morphologically very similar to *R. appendiculatus* but its ecology differs in that it is found in hotter and drier areas. *R. zambeziensis* is also more efficient at transmitting buffalo-derived *T. parva* infections, which commonly produce very low piroplasm parasitaemias (Blouin & Stoltz 1989). *R. duttoni* which is better known by its common name, Angolan Brown Ear Tick, is also a vector for *T. parva*, and is common in Angola (Gomes and Wouters 1991).

2.7. Control of theileriosis

2.7.1. Chemical control of ticks

Between the late 1890s and early 1940s, farmers used arsenic dips to protect their cattle against ticks and tick-borne diseases (TBDs). This went well until researchers realized that ticks were developing resistance to the chemical, making the dips inefficient. Subsequently, farmers moved over to organochlorines but these products also need to be changed periodically to avoid the development of acaricide resistance (George et al. 2004). Amidines and pyrethroids are the most commonly used acaricides. The dip tank systems have been adopted in many southern African countries and are the pillars for the surveillance and control of many livestock diseases. The use of acaricides is still the most prevalent way of controlling TBDs in livestock (Di Giulio et al. 2009).

Research done by Walker (2013) demonstrated that most models of tick-borne pathogens only focus on livestock when it comes to the dynamics of the disease and no focus is given to the role of wild ruminants. To fill this gap, they constructed a model that combined the use of acaricides with the presence of wild and domestic hosts. The model indicates a reduced effect of acaricides in the tick hosts but also indicates that the presence of wild hosts contributes to tick persistence. These results

indicate that in areas of interaction between wildlife and cattle, when livestock are the only target within a control strategy, ectoparasites are likely to remain a problem, and the continuous use of acaricides which will be needed, might have significant detrimental effects on the economy and the environment.

2.7.2. *Biological control of ticks*

Research is underway in the use of entomopathogenic fungi for biological control of different tick species. Because of the negative effects of chemical agents (environmental contamination, toxic effects to animals and the appearance of resistance of ticks to these chemicals), researchers are looking at a different means of controlling ticks. Studies on *Rhipicephalus* ticks specifically have already shown promising results when conidial formulations of *Beauveria bassiana* and *Metarhizium anisopliae* fungi were used to treat them (Kirkland et al. 2004; Reis et al. 2008). The main physiopathological mechanisms of the fungi are the following: adhesion of spores to the host cuticle, where they germinate, followed by penetration into the host, mycelia proliferation in the host's hemocoel and finally, the production of toxic metabolites that kill the tick host (Fernandes et al. 2012). A problem encountered with this control method is that different environmental factors such as temperature, ultraviolet (UV) radiation and desiccation influence the efficacy of the fungi (Rangel et al., 2004; Braga et al., 2002). Research is needed to produce fungi which will be more resistant to specific environments.

2.7.3. *Immunization*

Various immunization methods have been developed to protect cattle against ECF. Immunization can be achieved through the infection and treatment method: the animal is simultaneously infected with the parasite while it is treated chemoprophylactically with an agent that acts during the early stages of the disease. This was done by inoculating the cattle with an infective stabilate of the appropriate *T. parva* strains followed by treatment with oxytetracyclines (Radley et al. 1975). Evidence suggested that the severity of the ECF reactions in animals depended on the infection dose levels of the parasite, but when a group of cattle were tested with the same dosage of parasites a variety of reactions were seen and this method was ruled to be unsuitable for use as immunization (Radley, 1981).

In a study performed by Dolan (1980), cattle that were treated with the stabilate did not die from *T. parva* infection, while three quarters of the susceptible cattle died from the introduced *T. parva* infection. Oxytetracycline is believed to interfere with the maturation of sporozoites after infection (Spooner 1990). A study done in 2005 revealed that long-acting tetracyclines also have some efficacy in protecting animals from *Babesia* parasites (Kahn et al. 2005).

In 1982, a massive infection and treatment immunization programme was undertaken in the eastern province of Zambia. More than 50 000 calves were vaccinated by the infection and treatment method using long-acting tetracyclines. In the southern province the "Muguga cocktail" was used (Nambota et al. 1994). As a result, a reservoir of carriers was created through years of chemotherapy treatment and immunization of Zebu cattle in the eastern province of Zambia (Billiouw et al. 1999). Therefore, in order to avoid the development of carrier animals and potential transmission to cattle that could lead to a subpopulation of *T. parva* better adapted to the cattle host, the immunization and treatment of cattle for Corridor disease is prohibited in South Africa (Potgieter et al. 1985). Treatment of cattle during outbreaks is also prohibited; chemotherapeutic drugs are, therefore, not registered in South Africa.

Other attempts included the immunization of cattle with recombinant DNA vaccine constructs containing the Tp1, Tp2, Tp4, Tp5, and Tp8 antigens which helped with the overexpression of T lymphocyte production in the cattle (Mwangi et al. 2011). By introducing bovine Flt3L and granulocyte/macrophage colony stimulating factor (GM-CSF) prior to immunization, researchers succeeded in increasing the *T. parva*-specific proliferation, and IFN- γ -secreting CD4⁺ and CD8⁺ T cell responses in these cattle. But the cytotoxic T lymphocytes specific to the *T. parva* antigen were still absent (Mwangi et al. 2011).

A candidate for a sporozoite vaccine was tested in the form of a fusion protein containing a non-structural gene of the influenza virus A (NS1) and a recombinant p67 antigen gene, formulated in saponin, which gave rise to the development of anti-p67 antibodies that gave the animals protection against sporozoite infection (Musoke et al. 1992). A second attempt was made to increase the strength of this candidate (Nene et al. 1995). These authors found that certain characteristics of the native p67 were absent in fusion proteins (Musoke et al. 1992). They believed that by producing a more native p67 form it would incur a better response. They used a baculovirus expression system in insect cells to produce NS1-p67 fusion proteins. These fusion proteins also produced anti-p67 antibodies in animals but no antigen-specific T cells were produced in the peripheral blood. They concluded that the bacterially expressed antigen fusion was superior.

Immunization proves to be an effective method of controlling *T. parva*, but research in Tanzania showed that when farmers used immunization as protection for their animals, they tend to use less acaricides, which then gives rise to an increase in other TBDs over time (Lynen et al. 2012). More research is needed in the fields of immunization, sporozoite vaccine and the combined effects of acaricides and immunization and also in more efficient ways to communicate and raise awareness among local rural farmers on how to improve tick control.

2.7.4. Chemotherapy

The first successful reported use of chemotherapy on ECF in cattle was in the 1950s with the use of pamaquin (Neitz, 1950). Subsequently, analogues of menoctone and parvaquone were successful at treating ECF in infected cattle; buparvaquone was found to be more efficient at lower dosages. However, researchers were unable to confirm if treated cattle were completely cleared of *T. parva* or if they remained carriers (Dolan, 1986). Due to this, the use of buparvaquone is prohibited in South Africa.

Tetracyclines were first described in Neitz (1953; 1957) in the form of chlortetracycline and oxytetracycline, where it gave some protection to cattle against ECF. Wilde (1967) concluded that tetracycline, with little therapeutic activity, should be administered during the incubation period of the disease, where it seems to be the most effective.

Halofuginone lactate was also used to treat cattle with a 100% success rate in earlier cases (Schein and Voigt, 1979). Researchers from a different study (Njau et al. 1985) concluded that with early detection of the outbreak, the disease can effectively be controlled using halofuginone lactate. But, more recent research has shown that halofuginones are inactive against the early infection stages of the parasite (Dolan et al. 1988).

In recent years, Lizundia and colleagues (2008) identified 18 targets for chemotherapeutic action in other apicomplexans than *T. parva*. From these 18 targets, only 9 were identified to have clear matching sequences with *T. parva*. These putative inhibitors include: ciprofloxacin (inhibits *Theileria*-infected B cell growth) and three other compounds that inhibit *Theileria*-infected lymphocyte proliferation: fenoxaprop, triclosan and rifampin (Lizundia et al. 2008).

2.8. Laboratory diagnosis

In South Africa, several hundred game farms breed buffalo in captivity or semi-free ranging conditions. Specific conditions are in place and must be adhered to any time those animals are translocated. Buffalo are tested before they are translocated to a new disease-free area, and when they arrive at their new destination. For every movement, buffalo must be tested for foot-and-mouth disease virus, Corridor disease, bovine brucellosis and tuberculosis because these diseases can be transmitted to cattle. Two negative tests for Corridor disease conducted within a minimum of thirty days are a requisite to prove that they are *T. parva* free (Davies and Hofmeyr 2003). The identification of the parasite can be achieved in different ways, including serological methods such as indirect antibody test (IFAT) and enzyme-linked immunoabsorbent assay (ELISA), and more recently molecular methods such as quantitative real-time PCR (Davies and Hofmeyr 2003).

2.8.1. Conventional methods

The use of microscopes to examine lymph and blood smears for the presence of *T. parva* can be used for early identification. In blood smears parasites can be identified by morphological characteristics and by the use of certain staining methods: acridine orange staining method, Giemsa-staining and quantitative buffy coat method (Winters, 1967; Morzaria et al. 1992; Morzaria et al. 1999). This diagnostic procedure has a few limitations including that piroplasms and schizonts from *T. parva* are very difficult to distinguish from other *Theileria* species, if they should occur in the same blood/lymph sample (Norval et al. 1992). Also, parasites in carrier individuals are at such a low level that it is not always possible to detect them by microscopic examination. Therefore, this method can provide many false negative results and in the best of cases, it can identify the genus but not the species of *Theileria*.

2.8.2. Serological methods

The IFAT was originally developed to use piroplasm antigens for the detection of *T. parva* antibodies, but currently schizont antigens are used (Burrige et al. 1971; Burrige et al. 1973). The method, although good in many ways, has many limitations which include complications when it comes to standardization (Norval et al. 1992). There is a chance that antibodies in the animal have diminished although the parasite is still present in carrier animals (false negative), and on the other hand, antibodies can be detected where the parasites are no longer present (false positives). Therefore, IFATs alone are not sufficient to identify disease presence in carriers (Young et al. 1986; Bishop et al. 1992). Research done on a farm in the Ladysmith district suggested that false-positive IFATs from calves were obtained because of the presence of maternal-derived antibodies and that many of the IFAT positive reads were because of cross-reactions with *T. taurotragi* (Billiouw et al. 2002; OIE manual, 2004). Nevertheless, IFAT is routinely used in South Africa for *T. parva* antibody detection in “disease free” buffalo (Schindler et al. 1969; Laubscher and Hoffman, 2012).

ELISA tests use recombinant polymorphic immunodominant molecules (PIM) to detect *T. parva* antibodies (Katende et al. 1998). ELISA tests are more sensitive and specific than IFAT. Sensitivity of ELISA and IFAT was both reported to be 90% but ELISA had a higher specificity (90%) than IFAT (80%) (Muraguri et al. 1999). Despite this higher performance, ELISA still has its limitations: as happens with IFAT, antibodies may be at low levels in an animal which has cleared the infection, but the test gives a positive result because ELISA does not detect the parasite but only the presence of antibodies (Bishop et al. 1992 and Dolan 1986).

2.8.3. Molecular methods

Because of the limits of conventional and serological methods, researchers have developed molecular diagnostic assays for the identification of *T. parva*. These molecular techniques include conventional

PCR assays, PCR-based hybridization and restriction fragment length polymorphisms (RFLP), real-time PCR assays and reverse line blot (RLB) hybridization assays. We shall concentrate on RFLP, real-time PCR and the RLB hybridization assay because these techniques will be used in the project.

Based on its high sensitivity, quantitative real-time PCR (qPCR) detection is the preferred diagnostic tool (Bustin *et al.* 2009). It has the ability to distinguish between different species in a sample through the use of fluorescent hybridization probes and a melting curve analysis. qPCR collects information in the exponential growth phase and not at the end-phase; therefore, because the number of amplicons generated is proportional to the fluorescent signal, qPCR is able to detect and quantify the parasite DNA in a sample (Nicolas *et al.* 2002). The *T. parva* specific real-time PCR (Sibeko *et al.* 2008), has the ability to distinguish between *T. parva* and other blood parasites in a sample with as low as $8.79 \times 10^{-4}\%$ parasitaemia. This is done by the inclusion of a primer set that amplifies *T. parva* DNA and *Theileria* sp. (buffalo); the hybridization probes yield a specific melting peak for *T. parva*. A set of primers that is specific to the genus *Theileria* can also be included to detect other *Theileria* species present in the sample. The second hybridization probe set hybridizes to the amplicons to make it possible to visualize *Theileria* positive samples through fluorescence. Samples are visualized at two different wavelengths: 705 nm for *Theileria* group-specific hybridization and 640 nm for *T. parva* hybridization. Peaks visualized on the graphs of the 640 and 705 nm tests indicated the presence of *T. parva* and *Theileria* species, respectively (Sibeko *et al.* 2008).

The reverse line blot (RLB) hybridization assay uses multiple probes to simultaneously detect PCR products amplified from different disease agents in one sample (Gubbels *et al.* 1999). It has been shown to detect *T. annulata* with a parasitaemia as low as $4.8 \times 10^{-5}\%$. The RLB hybridization assay makes use of PCR amplification and hybridization to deliver a much higher level of sensitivity than conventional PCR assays. It is used to identify different species of *Babesia*, *Theileria*, *Ehrlichia* and *Anaplasma*. Also, in the detection process chemiluminescence is used instead of radioactivity, making it less dangerous (Anonymous, 2004). This method is a cost-effective way to identify multiple parasites in one test and has been successfully used in the past to identify *Theileria*, *Babesia*, *Anaplasma* and *Ehrlichia* species in blood samples from different infected ruminants (Gubbels *et al.* 1999; Bekker *et al.* 2002).

2.9. Differentiation of *Theileria parva* isolates

Traditionally, differentiation between *T. parva* isolates was done on the basis of the epidemiology of the disease and on the number of schizonts and piroplasms observed in blood smears of infected animals (Norval *et al.* 1992). Serologically, a battery of monoclonal antibodies (Mabs) has been used against various antigenic genes to define the Mab reactivity profiles of the different isolates (Minami *et al.*, 1983; Irvin *et al.*, 1983; Conrad *et al.* 1987b; Conrad *et al.* 1989). In PCR-based assays, RFLP

profiles have been used to discriminate between different *T. parva* isolates by comparing the variable region of the parasite antigen genes (Allsopp et al. 1989; Bishop et al. 1993; Conrad et al. 1987b; Conrad et al. 1989; Geysen et al. 1999; Oura et al. 2003).

2.10. Antigenic genes

2.10.1. *p67*

p67 is a sporozoite antigen gene that is expressed in the sporozoite stage of the life cycle (Iams et al. 1990, Nene et al. 1996). Recent work suggests that *p67* plays an important role in parasite entry into host lymphocytes (Toye et al. 2014). In previous studies, the absence or presence of a specific 129 bp sequence in the central region of the gene was successfully used to distinguish between cattle-derived (absence) and buffalo-derived (presence) *T. parva* in East Africa (Nene et al. 1992, Nene et al. 1996). Research done by Collins (1997) identified both of these alleles in *T. parva* isolates from buffalo from the Kruger National Park in South Africa, leading to the conclusion that using only these two alleles to differentiate between buffalo-derived and cattle-derived *T. parva* is not possible.

Sibeko (2010) also identified two new alleles for the *p67* gene apart from the allele containing the 129 bp deletion (allele 1) and the one without it (allele 2). The new alleles are characterized by containing a different 174 bp deletion (allele 3) and an allele without the 174 deletion (allele 4) but a similar sequence to allele 3 (Sibeko et al. 2010).

2.10.2. *p104*

The 104 kDa antigen of *T. parva* is encoded by a single copy gene and can also only be observed in the sporozoite stage of the *T. parva* life cycle (Iams et al. 1990). Previous studies have found that the *p104* RFLP profiles obtained from buffalo-derived parasites are more polymorphic than those found in cattle and contain no significant deletion (Geysen *et al.* 1999). This observation has been used to distinguish between cattle and buffalo-derived *T. parva*. Four *p104* alleles have been identified previously in East Africa (Skilton et al. 2002) and they are subdivided into two clades, A and B. Allele 1 represents *T. parva* Muguga (cattle-derived) *p104* gene sequence, allele 2: Marikebuni (cattle-derived) and Uganda (cattle-derived) *T. parva* stocks, allele 3: *T. parva* Boloni (cattle-derived) *p104* gene sequence and 4 represents 7014 (buffalo-derived) *p104* gene sequence. Further phylogenetic analysis of *p104* sequences identified in South Africa led to the formation of clades A and B which could be divided into sub-clades: A1, A2, B1 and B2. Alleles 1 and 4 were found in B1 where it would seem that they evolved from a common ancestor, alleles 2 and 3 were grouped together in clade A2 and novel *p104* sequences were located in subclades A1 and B2 (Sibeko et al. 2011). This work identified a high sequence similarity amongst the buffalo-derived and cattle-derived isolates and could not be used to better differentiate between them.

2.10.3. PIM

The PIM gene codes for the Polymorphic Immunodominant Molecule (PIM), a *T. parva* antigen found both in sporozoites and schizonts (Katende et al. 1998). Evidence suggests that PIM has a function in the establishment of the parasite within the host lymphocytes. PIM has been found to be expressed when the parasite is entering the host lymphocytes at which time it is located on the parasite surface (Toye et al. 2014). This antigen has been characterized in the sporozoite and schizont stages of the *T. parva* life cycle but predominantly in the schizont stage and thus, is believed to play a role as a target antigen in the induction of the cytotoxic T cell response. As with the p104 gene, PIM PCR-RFLP profiles from buffalo are more polymorphic than those from cattle. Previous work suggests that this gene has an extremely high recombination rate because all isolate sequences tested were different (Toye et al. 1995, Sibeko et al. 2011). Four alleles for PIM have been identified in South Africa: i) a cattle type, which exclusively contains the regions between nucleotides 29-259 and 493-497, ii) a buffalo type, containing a specific 20 amino acid insert and 2 amino acid motifs and iii) two mixed types (M1 and M2) containing both cattle and buffalo type sequences. M1 isolates contain cattle type sequences at the amino-terminus and buffalo type sequences at the carboxyl-terminus. In M2, the positions of the buffalo and cattle type sequences are reversed (Sibeko et al. 2011). Her work indicated that *T. parva* samples originating from cattle had more mixed PIM sequences than those originating from buffalo. Also, in conjunction with other molecular tools (micro- and minisatellite markers) more reliable and specific markers that could be used to differentiate between the different *T. parva* disease syndromes should be studied in detail.

2.11. Other disease syndromes of importance in buffalo and cattle

2.11.1. Bovine anaplasmosis

Another important disease of the livestock industry, with high morbidity and mortality levels in cattle is bovine anaplasmosis (Kocan et al. 2003). The disease is caused by *Anaplasma marginale* and the less virulent *A. marginale* subsp. *centrale*, which induces a milder form of the disease. The parasite is transmitted biologically by ticks (*Rhipicephalus decoloratus*, *R. microplus*, *R. evertsi evertsi*, *R. simus* and *Hyalomma rufipes* in South Africa) and mechanically by insects (De la Fuente et al. 2005; Dreyer et al. 1998; Potgieter and van Rensburg, 1987; Potgieter and Stoltz, 2004). *A. marginale* (Theiler, 1909) and *A. marginale* subsp. *centrale* (Theiler, 1911) were first described in South Africa and *A. marginale* has also been identified in tropical and subtropical areas around the world including Africa, Europe, the Caribbean, Middle and Far East, North and South America (Kocan et al. 2003). Clinical symptoms for this disease include weakness, constipation, high fever, laboured breath, rapid weight loss and jaundice due to erythrocyte destruction. Symptoms are more severe in adults than young

animals; infected calves that are one year or younger usually show no symptoms but become carriers of the disease (Richey, 1992). *Anaplasma* parasites have previously been identified in the following animals: cattle (*Bos taurus*), water buffalo (*Bubalus bubalis*), bison (*Bison bison*; *B. bonasus*), African antelopes, white-tailed deer (*Odocoileus virginianus*) and mule deer (*Odocoileus hemionus*) (Kocan et al. 2003). It has been shown that except for two cases of acute anaplasmosis in giraffes, *A. marginale* has no clinical effect on wild ruminants (Augustyn and Bigalke, 1974; Davidson and Goff, 2001). With this said, wild animals that are persistently infected with *Anaplasma* parasites can become reservoirs for these parasites, including the African buffalo which already serves as a reservoir for other important haemoparasites (Kocan et al. 2003).

2.11.2. Heartwater

Heartwater (cowdriosis) is an acute, infectious, non-contagious, fatal, tick-borne rickettsial disease which is caused by *Ehrlichia ruminantium* (Provost and Bezuidenhout, 1987; Cowdry, 1925). The parasite is transmitted to susceptible animals by ticks of the genus *Amblyomma*, primarily *A. hebraeum* and *A. variegatum* (Cowdry, 1925; Walker and Olwage, 1987; Allsopp et al., 2006). The disease has been identified in all sub-Saharan countries and several islands in the Indian Ocean (Comoros Islands, Madagascar, Mauritius, Reunion, Zanzibar), on the Atlantic Coast (Sao Tomé) and in the Caribbean (Antigua, Guadeloupe and Marie-Galante) (Perreau et al. 1980). Clinically, the disease is seen in four different forms: peracute, acute, subacute or mild/subclinical infections. Symptoms include fever, respiratory distress, hyperesthesia, lacrimation and in some cases diarrhea. Heartwater has rarely been found to cause mortality in African buffalo, blesbok (*Damaliscus albifrons*), the black wildebeest (*Connochaetes gnu*), the eland (*Taurotragus oryx oryx*), giraffe (*Giraffe camelopardalis*), greater kudu (*Tragelaphus strepsiceros*), sable antelope (*Hippotragus niger*), sitatunga (*Tragelaphus spekii*), steenbok (*Raphicerus campestris*), and lechwe (*Kobus leche kafuensis*) (OIE manual, 2007). These species may play roles as reservoirs for the parasite.

2.11.3. Bovine babesiosis

Babesia is believed to be the second most common blood parasite after trypanosomes, representing a significant health risk for cattle (Hunfeld et al., 2008). Also known as redwater, babesiosis is classified as a tick-borne disease that affects cattle and has a severe impact on the milk and meat production industries (Bock et al. 2004; OIE manual, 2008). This disease being zoonotic also has implications to humans worldwide (Gray et al. 2010). Important species of *Babesia* that cause disease include *Babesia bovis* and *B. bigemina* (both transmitted by *Rhipicephalus microplus*, with *B. bigemina* also being transmitted by *R. decoloratus*) in cattle while *Babesia divergens* and *Babesia microti* (both possibly transmitted by *Ixodes ricinus*) cause disease in humans (Gray et al. 2010). The disease follows the distribution of its arthropod vector, in this case: Africa, Asia, central and South America and Australia (OIE manual, 2008). Symptoms of the disease include: anemia, fever,

hemoglobinuria, marked splenomegaly and in some cases death (Bock et al. 2004; OIE manual, 2008). Cattle act as reservoir host for *Babesia bovis* and *B. bigemina* (Hunfeld et al. 2008) and when they come into contact with wild ruminants they may be able to transmit the parasites through a tick vector to them. Previous work by Karbe et al. 1979, showed that it was possible to establish a carrier state in two buffalo through sub-inoculation of infected blood into splenectomized calves.

2.12 References

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Chapter 3

Identification of *Theileria parva* and other tick-borne haemoparasites in African buffalo (*Syncerus caffer*) from northern Botswana and Zimbabwe

3.1. Abstract

The African buffalo (*Syncerus caffer*) is a host for many pathogens known to cause economically important diseases and is often considered an important reservoir for livestock diseases. Theileriosis, heartwater, babesiosis and anaplasmosis are considered the most important tick-borne diseases of livestock in sub-Saharan Africa, resulting in extensive economic losses to livestock farmers in endemic areas. Information on the distribution of tick-borne diseases and ticks is scarce in northern Botswana and southern Zimbabwe. Nevertheless, this data is necessary for targeting surveillance and control measures in livestock production at national level. In order to address this gap, we analyzed 160 blood samples from buffalo herds for the presence of common tick-borne haemoparasites causing disease in livestock, collected in two of the main wildlife areas of northern Botswana: the Chobe National Park (CNP, n=64) and the Okavango Delta (OD, n=56) as well as from the Gonarezhou National Park (GNP, n=40) in Zimbabwe. Analysis of the reverse line blot (RLB) hybridization assay results revealed the presence of *Theileria*, *Babesia*, *Anaplasma* and *Ehrlichia* species, either as single or mixed infections. Among the *Theileria* spp. present, *T. parva* (60%) and *T. mutans* (37%) were the most prevalent in the two wildlife areas from Botswana, while *Theileria* sp. (sable) (50%), *T. parva* (48%) and *T. mutans* (38%) were most prevalent in GNP, Zimbabwe. Other species of interest were *Anaplasma marginale* subsp. *centrale* (30%), *A. marginale* (20%), *Babesia occultans* (23%) and *Ehrlichia ruminantium* (6%) in Botswana and *Anaplasma marginale* subsp. *centrale* (25%) and *Babesia occultans* (15%) in Zimbabwe. The indirect fluorescent antibody test (IFAT) indicated 74% of the Botswana samples to be positive for the presence of *T. parva* antibodies. Quantitative real-time PCR (qPCR) detected the highest level of animals infected with *T. parva* (81% in Botswana and 70% in Zimbabwe). The level of agreement between the tests for detection of *T. parva* positive animals

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was higher between qPCR and IFAT ($\kappa=0.56$), than between qPCR and RLB ($\kappa=0.26$) or the latter and IFAT ($\kappa=0.15$). This is the first report of tick-borne haemoparasites in African buffalo from northern Botswana and one of only a few from Zimbabwe. Overall, animals from the CNP showed higher levels of infection than those from GNP and OD, except for *Theileria* spp. Considering the absence of fences separating wildlife and livestock in the CNP and to a lesser extent, GNP, and the higher levels of some parasite species in buffalo from that area, surveillance of tick-borne diseases in livestock at the interface in the CNP should be prioritized.

3.2. Introduction

Theileriosis, babesiosis, anaplasmosis and heartwater are considered to be the most important tick-borne diseases (TBDs) of livestock in sub-Saharan Africa, resulting in extensive economic losses to farmers in endemic areas. The African buffalo (*Syncerus caffer*) is the natural reservoir host of *Theileria parva*, which is transmitted by the tick species, *Rhipicephalus appendiculatus*, *R. zambeziensis* and *R. duttoni* (Norval et al. 1991; Uilenberg, 1999). *T. parva* causes East Coast fever (ECF), which occurs in eastern and central Africa. ECF was introduced into southern Africa in the early 1900s through cattle importation from East Africa and was eradicated from South Africa in the 1950s (Latif and Hove, 2011; Lawrence, 1979). *T. parva* also causes Corridor disease, which is still prevalent in South Africa in areas where buffalo and cattle share grazing grounds in the presence of its tick vectors. It is a controlled disease in South Africa because of a concern that ECF might recur (Potgieter et al. 1988; Stoltsz, 1989). Although *T. parva* distributions have been described in Mozambique (Chaisi et al. 2011), Zambia (Munang'andu et al. 2009) and Zimbabwe (Latif and Hove, 2011), no information on the distribution of *T. parva* is available for many other southern African countries, including Botswana.

In addition to *T. parva*, buffalo are also thought to be the original reservoir host of other non-pathogenic, mildly pathogenic and benign *Theileria* species namely, *Theileria mutans*, *Theileria velifera*, *Theileria buffeli*, *Theileria* sp. (buffalo) (Norval et al. 1992; Stoltsz, 1996) and *Theileria* sp. (bougasvlei) (Zweygarth et al. 2009; Mans et al. 2011). *Theileria* parasites usually occur as mixed infections in buffalo and cattle. Although the benign and non-pathogenic forms do not have any significant economic importance, their presence could interfere with the interpretation of results obtained in some diagnostic tests designed to diagnose the pathogenic *T. parva*. Diagnostic tests for *T. parva* include microscopic examination of blood smears for the presence of piroplasms and schizonts and serological methods such as the indirect fluorescent antibody test (IFAT) which is routinely used in South Africa for *T. parva* antibody detection in “disease free” buffalo (Schindler et al. 1969; Laubscher and Hoffman, 2012). Molecular diagnostic methods detect specific parasite sequences in DNA extracts from blood or tissue samples. The reverse line blot (RLB) hybridization assay makes use of polymerase chain reaction (PCR) amplification of haemoparasite small subunit ribosomal RNA

genes (srRNA) which are screened with group- and species-specific probes for the simultaneous detection and identification of haemoparasites in mixed infections (Gubbels et al. 1999). To date, the most sensitive molecular test for the detection of *T. parva* is a quantitative real-time PCR (qPCR) using hybridization probe chemistry, where the central region of the parasite 18S rRNA gene is amplified and the presence of *T. parva* is confirmed by melting curve analysis (Sibeko et al. 2008). It is currently routinely used to test for *T. parva* infections in buffalo and cattle in South Africa as part of the Corridor disease control strategy.

It has been shown that African buffalo are also carriers of a number of other tick-borne parasites which are detrimental to livestock including *Ehrlichia ruminantium*, *Babesia bigemina*, *B. bovis*, *Anaplasma marginale* and *A. marginale* subsp. *centrale* (Andrew and Norval, 1989; Iseki et al. 2007; Kuttler, 1965). Although buffalo show no disease symptoms, as reservoir hosts, they may represent a threat to the livestock industry. *Ehrlichia ruminantium*, an intracellular rickettsial bacterium, is the causative agent of heartwater (cowdriosis) and is transmitted by three-host ticks belonging to the genus, *Amblyomma* (Allsopp, 2010). *Babesia bigemina* and *B. bovis* cause bovine babesiosis, commonly known as redwater fever. Tick vectors for these parasites include *Rhipicephalus microplus* (formerly *Boophilus microplus*) and *Rhipicephalus annulatus* (formerly *Boophilus annulatus*). It is believed that *Babesia* is the second most common blood parasite after trypanosomes representing a significant health risk for cattle (Hunfeld et al. 2008). *Anaplasma marginale* causes bovine anaplasmosis which is characterized by the infiltration of the host's red blood cells. It can be transmitted to other hosts through mechanical transmission but the most important mode of transmission is via tick bites, the main tick vector being *R. decoloratus* (formerly *Boophilus decoloratus*) (Potgieter and van Rensburg, 1987; Potgieter and Stoltz, 2004). *Anaplasma marginale* subsp. *centrale* causes a milder form of anaplasmosis, and is used in a live blood vaccine in many countries, including South Africa (Potgieter and van Rensburg, 1983).

Generally speaking, publications on significant tick-borne haemoparasites in Botswana are limited (Carmichael and Hobday, 1975; Sharma et al. 2003; Batisani et al. 2012; Mahabile, 2013) and there are no published reports of the presence of *T. parva* in livestock in this country. In addition, published literature on the occurrence of pathogens in buffalo populations from Botswana is very scarce (Jori et al. 2013). Publications in Zimbabwe are also limited (Latif and Hove, 2011). Therefore, the goal of this study was to determine the prevalence of tick-borne parasites circulating in two distinct buffalo populations from northern Botswana and one from Zimbabwe using different diagnostic methods and to use these data to compare the performance of those tests in detecting *T. parva* in buffalo.

3.3. Materials and Methods

3.3.1. Buffalo sampling

Botswana: The Chobe National Park (CNP) and Okavango Delta (OD) are located in two different districts of northern Botswana (Chobe and Ngamiland Districts, respectively) and represent the largest wildlife areas in this part of the country. They are both integrated in the Foot and Mouth Disease infected area, a large part of the northern region of Botswana devoted to wildlife conservation in which buffalo populations are separated from the primary cattle export and buffer zones by the use of veterinary cordon fences (Figure 1). The Chobe, Zambezi and Okavango rivers are the largest in the region, providing abundant water throughout the year. Rainfall is strongly seasonal, occurring mostly from December to April (wet season). Vegetation consists mainly of deciduous dry woodland and scattered grasslands. Wildlife abundance is fundamentally dependent on rainfall and water availability and varies cyclically throughout the years (Alexander et al. 2012). Herbivores that are located in the park include: African elephant (*Loxodonta africana*), hippopotamus (*Hippopotamus amphibius*), African buffalo (*Syncerus caffer*), bushbuck (*Tragelaphus scriptus*) plains zebra (*Equus quagga*), roan antelope (*Hippotragus equinus*), sable antelope (*Hippotragus niger*), red lechwe (*Kobus leche leche*) and impala (*Aepyceros melampus*) (<http://www.chobenationalpark.co.za/travel-info/fauna-and-flora>). The CNP encompasses 10 700 km² of savannah grassland. The boundaries of CNP are natural, the Chobe river in the north constituting the natural border between Botswana and Namibia. There is no physical barrier preventing contacts between cattle and wildlife and the main water source for the animals in that area is the Chobe river which is exposed to seasonal variations of water levels. The OD encompasses 16 000 km² and contrary to the CNP, it is delineated from livestock areas by a double veterinary cordon fence to prevent contacts between wildlife and cattle (Jori et al. 2013), and it is largely flooded throughout the year. Herbivores found in OD include: African elephant, hippopotamus, African buffalo, bushbuck, waterbuck (*Kobus ellipsiprymnus*) plains zebra, roan antelope, sable antelope, Red Lechwe, kudu (*Tragelaphus imberbis*), tsessebe (*Damaliscus lunatus*) and impala (<http://www.okavangodeltasafaris.com/wildlife.html>). According to the last available wildlife census from northern Botswana, buffalo populations and densities are estimated at 31 500 individuals and 0.94 individuals/km² in OD and 7 500 individuals and 0.23 individuals/km² in the CNP (Chase, 2011).

The sampling process was opportunistic and details of the capture approach were described (Jori et al. 2013). During the capture process, blood samples were collected from a total of 120 individual buffalo. In the CNP, buffalo were captured along the Chobe river and blood samples were collected from 64 individual buffalo from 7 distinct herds. In the OD, 8 buffalo herds were sampled in two different management units: the Moremi Game Reserve (MGR) (n=18 individuals, 4 herds) and in the NG30 area (n=38 individuals, 4 herds) (Figure 1). Buffalo densities in those locations were estimated

at 1.88 buffalo/km² for the Chobe river, 1.37 buffalo/km² for the MGR and 3.55 buffalo/km² for the NG30 area of the OD (Chase, 2011). The sex and age of the animals were recorded. Age was measured according to dentition; animals younger than 3 years were considered as young and animals older than 3 years were considered to be adults. Whole blood samples were obtained from the jugular vein, maintained in refrigeration and sent at the Botswana National Veterinary Laboratory in Gaborone. There, they were centrifuged at 3500 rpm for 15 minutes. Sera was then harvested with a pipette and stored frozen at -20°C, until the samples were ready to be sent to the Agricultural Research Council–Onderstepoort Veterinary Institute (ARC-OVI) in South Africa for analysis.

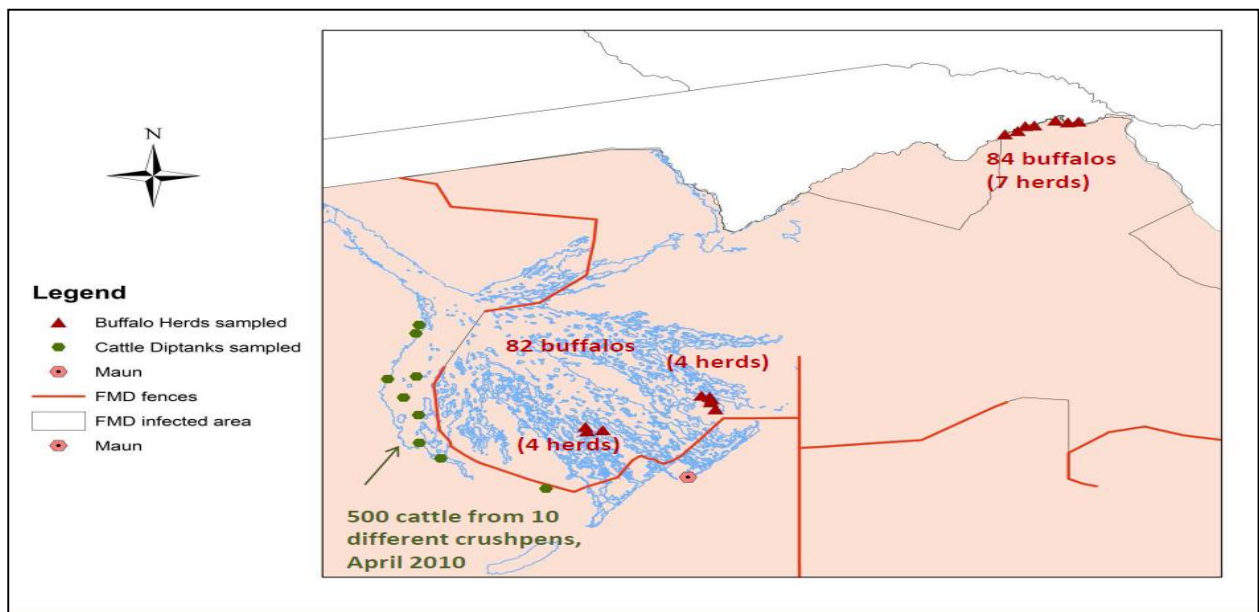


Figure 1: Schematic representation of where samples were collected from in northern Botswana.

Zimbabwe: The Gonarezhou National Park (GNP) is situated in the south-eastern part of Zimbabwe, in the Masvingo Province (Figure 2). With a size of 5 053 km², it is the second largest game reserve in Zimbabwe after the Hwange National Park (Gandiwa et al., 2013). This park, along with the Kruger National Park and the Limpopo National Park in South Africa forms part of the Great Limpopo Transfrontier Park. Three rivers flow through the Park: the Save, Runde and Mwenezi rivers, which form a natural oasis for a diverse range of bird, wildlife and fish species which gather to feed and drink. The park, like CNP, experiences wet summers where rainfall is abundant between November and April. A variety of large herbivores can be found in this park: African elephant, hippopotamus, African buffalo, giraffe (*Giraffa camelopardalis*), plains zebra, waterbuck, roan antelope, sable antelope, blue wildebeest (*Connochaetes taurinus*), eland (*Taurotragus oryx*), kudu, nyala (*Tragelaphus angasii*) and impala (Gandiwa, 2012). Between the early 1970s and the 1990s the park was enclosed with fences but most of these have been destroyed or removed through theft and vandalism (Gandiwa et al., 2013).

During the sampling process, blood was collected on filter paper from 40 individual buffalo from the Gonarezhou National Park. The sex and age of the animals or the geographical locations of the capture sites were not recorded when the blood was sampled. Buffalo densities in the park were estimated at 0.17 buffalo/km² between 2001 and 2009 (Gandiwa et al., 2013).

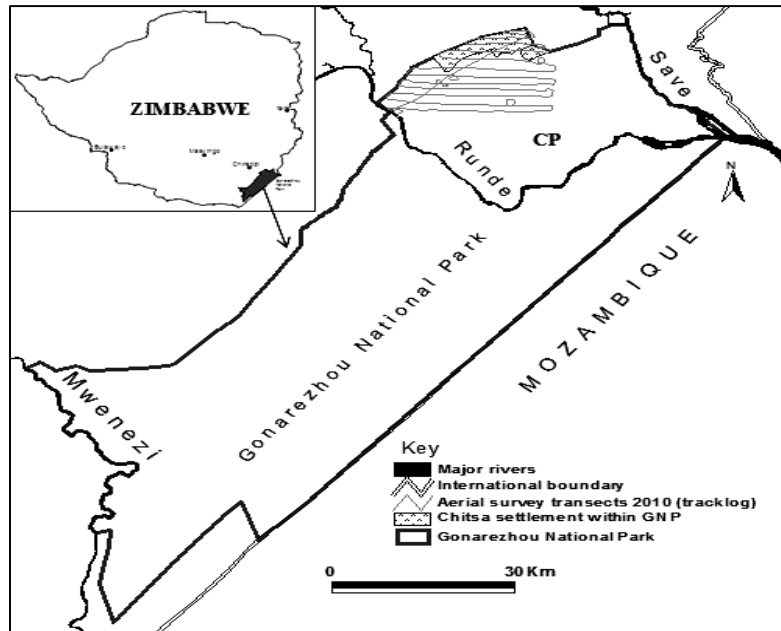


Figure 2: Map of Gonarezhou National Park (GNP) (Gandiwa et al. 2013).

3.3.2. Blood smears and DNA extraction

A total of 120 thin layer smears from Botswana were prepared in the field from a blood drop of the ear sublimine vein of the captured buffalo (two individuals were missed). Those were dried in the sun and fixed with methanol. At the laboratory, each smear was stained with Giemsa dye following the standard procedures. Genomic DNA was extracted from the Giemsa-stained slides using the QIAmp DNA mini kit (Qiagen) following the manufacturer's protocol. In GNP, blood was collected on FTA filter paper from 40 individual buffalo. Genomic DNA was extracted from the filter paper using the QIAmp DNA mini kit (Qiagen) following the manufacturer's protocol. The DNA for all samples were eluted into 100 µl of TE buffer and stored at -20°C until further use.

3.3.3. PCR amplification and reverse line blot (RLB) hybridization assay

A total of 160 DNA samples (120 from Botswana and 40 from Zimbabwe) were tested using the RLB hybridization assay as previously described (Chaisi et al. 2011; Gubbels et al. 1999). The V4 hypervariable region of the parasite 18S rRNA gene was amplified using primers RLB-F2 (5' GAC ACA GGG AGG TAG TGA CAA G 3') and RLB-R2 (Biotin label- 5' CTA AGA ATT TCA CCT CTG ACT 3') (Nijhof et al. 2005), while the V1 region of the parasite 16S rRNA gene was amplified from *Ehrlichia* and *Anaplasma* species using primers Ehr-F (5' GGA ATT CAG AGT TGG ATC

MTG GYT CAG 3') and Ehr-R (Biotin label-5' CGG GAT CCC GAG TTT GCC GGG ACT TYT TCT 3') (Bekker *et al.* 2002).

The PCR was prepared as follows: 5 µl DNA (30-50 ng), 12.5 µl Platinum Quantitative PCR SuperMix-UDG mix (Invitrogen, The Scientific Group, South Africa), and 20 pmol of each primer were made up to a total volume of 25 µl using nuclease-free water. Amplification was done using a touchdown PCR programme as previously described (Nijhof *et al.* 2003) and consisted of the following cycles: 3 minutes at 37°C; 10 minutes at 94°C; 2 cycles of 94°C for 20 seconds; 67°C for 30 seconds; 72°C for 30 seconds, decreasing from 67°C by 2°C every second cycle for five times. The annealing temperature continued to decrease till it reached 57°C, followed by 40 cycles of 94°C for 20 seconds; 57°C for 30 seconds and 72°C for 30 seconds, lastly 1 cycle of 72°C for 7 min. The 9600 Perkin-Elmer thermocycler (Applied Biosystems, South Africa) was used for these reactions. A *T. parva* positive buffalo DNA sample, 102 (Sibeko *et al.* 2008), and nuclease-free water were used as positive and negative controls, respectively. Amplicons were visualised on a 2% ethidium bromide-stained agarose gel and then screened by the RLB hybridization assay as previously described (Gubbels *et al.* 1999; Nijhof *et al.* 2005).

Theileria, *Ehrlichia*, *Anaplasma* and *Babesia* group- and species-specific oligonucleotide probes (Table 1) were diluted to a 200 to 1 600 pmol/150 ml concentration in 500 mM NaHCO₃ (pH 8.4) and were attached to the membrane with the amino-linker by adding the solution containing the probe to the slots of the miniblotter; incubation followed at room temperature for 1 min. The solutions were removed by aspiration, and the membrane was incubated in 100 ml of 100 mM NaOH at room temperature for 10 min. The membrane was washed in 125 ml solution of 203 SSPE–0.1% sodium dodecyl sulfate (SDS) (203 SSPE consists of: 360 mM NaCl, 20 mM NaH₂PO₄, and 2 mM EDTA [pH 7.4]) at 50°C for 10 min in a shaking incubator. On the day that the membrane was used, it was washed at 42°C for 5 min with a 125 ml solution of 2X SSPE–0.1% SDS. The membrane was placed in the MN45 miniblotter (Immunetics, Cambridge, Mass.) so that when the PCR products were loaded they would run across all the probes previously linked to the membrane. The PCR product was made up to 150 µl of 2X SSPE–0.1% SDS and heated to 99.9°C for 10 min, and afterwards the solution was cooled on ice immediately. Denatured PCR samples were applied to the membrane and incubated at 42°C for 60 min. PCR products were removed by aspiration, and the blot was washed twice in 125 ml of 2X SSPE–0.5% SDS at 42°C for 10 min inside a shaking incubator. Next, the membrane was incubated in a 10 ml solution of 1:4 000-diluted peroxidase-labeled streptavidin (Boehringer, Mannheim, Germany) in 2X SSPE–0.5% SDS at 42°C for 30 min. Afterward, the membrane was washed twice in 125 ml of 2X SSPE–0.5% SDS at 42°C for 10 min inside a shaking incubator. After two rinses in 125 ml of 2X SSPE for 5 min at room temperature, an incubation for 1 min in a 10 ml solution of ECL (ECL1 + ECL2) detection fluid (Amersham, Little Chalfont, Buckinghamshire,

United Kingdom) was followed by exposure to an X-ray film for 5 to 20 min by placing the membrane on the X-ray film inside a closed container. After the experiment, all PCR products were stripped from the membrane by washing the membrane twice in 1% SDS at 80°C for 30 min. If the membrane was not stripped, it was rinsed in 20 mM EDTA (pH 8.0) at 4°C and stored in a plastic bag for reuse (Gubbels *et al.* 1999).

Table 1: The oligonucleotide probes that were used in the RLB hybridization assays (Symbols indicate degenerate positions: R = A/G, W = A/T, K = G/T).

Target species	Probe sequence	Reference
<i>Ehrlichia/Anaplasma</i> genus-specific	GGGGGAAAGATTTATCGCTA	Schouls et al. 1999
<i>A. bovis</i>	GTAGCTTGCTATGAGAACA	Bekker et al. 2002
<i>A. marginale</i> subsp. <i>centrale</i>	TCG AAC GGA CCA TAC GC	Bekker et al. 2002
<i>A. marginale</i>	GACCGTATACGCAGCTTG	Bekker et al. 2002
<i>A. phagocytophilum</i>	TTGCTATAAAGAATAATTAGTG	Bekker et al. 2002
<i>Anaplasma</i> sp. <i>Omatjenne</i>	CGGATTTTTATCATAGCTTGC	Bekker et al. 2002
<i>E. canis</i>	TCTGGCTATAGGAAATTGTTA	Schouls et al. 1999
<i>E. chaffeensis</i>	ACCTTTTGGTTATAAATAATTGTT	Schouls et al. 1999
<i>E. ruminantium</i>	AGTATCTGTTAGTGGCAG	Bekker et al. 2002
<i>Babesia</i> genus-specific 1	ATTAGAGTGTTTCAAGCAGAC	Georges et al. 2001
<i>Babesia</i> genus-specific 2	ATTAGAGTGTTTCAAACAGGC	Georges et al. 2001
<i>B. bovis</i>	CAGTTTCGCCTGTATAATTGA	Gubbels et al. 1999
<i>B. bicornis</i>	TTGGTAAATCGCCTTGGTC	Nijhof et al. 2003
<i>B. bigemina</i>	CGTTTTTCCCTTTTGTGG	Gubbels et al. 1999
<i>B. caballi</i>	GTTGCGTTGTTCTTGCTTT	Butler et al. 2008
<i>B. canis</i>	TGCGTTGACCGTTGAC	Matjila et al. 2004
<i>B. divergens</i>	GTTAATATTGACTAATGTCGAG	Nijhof et al. 2003
<i>B. felis</i>	TTATGCTTTTCCGACTGGC	Bosman et al. 2007
<i>B. gibsoni</i>	TACTTGCCTTGTCTGGTTT	Yisaschar-Mekuzas et al. 2010
<i>B. leo</i>	TTATGCTTTTCCGACTGGC	Bosman et al. 2007
<i>B. major</i>	TCCCACTTTGGTTGGTGT	Georges et al. 2001
<i>B. microti</i>	GAATGATGCCGACTTAAACCCT	Nijhof et al. 2003
<i>B. occultans</i>	CCTCTTTTGGCCCATCTCG	He et al. 2011
<i>B. rossi</i>	CGGTTTGTGCTTTGTG	Matjila et al. 2004
<i>B. vogeli</i>	AGCGTGTTTCGAGTTTGCC	Matjila et al. 2004
<i>Babesia</i> sp. (sable)	GCGTTGACTTTGTGTCTTT	Oosthuizen et al. 2008

<i>Theileria/Babesia</i> genus-specific	TAATGGTTAATAGGARCRGTTG	Nijhof ^a (unpublished)
<i>Theileria</i> genus-specific	ATTAGAGTGCTCAAAGCAGGC	Nijhof ^a (unpublished)
<i>T. annae</i>	CCGAACGTAATTTTATTGATTG	Yisaschar-Mekuzas et al. 2010
<i>T. annulata</i>	CCTCTGGGGTCTGTGCA	Gubbels et al. 1999
<i>T. bicornis</i>	GCGTTGTGGCTTTTTTCTG	Nijhof et al. 2003
<i>T. buffeli</i>	GGCTTATTTTCGGWTTGATTTT	Gubbels et al. 1999
<i>T. equi</i>	TTCGTTGACTGCGYTTGG	Butler et al. 2008
<i>T. lestoquardi</i>	CTTGTGTCCCTCCGGG	Schnittger et al. 2004
<i>T. mutans</i>	CTTGCGTCTCCGAATGTT	Gubbels et al. 1999
<i>T. ovis</i>	TGCGCGCGGCCTTTGCGTT	Bekker et al. 2002
<i>T. parva</i>	GGACGGAGTTCGCTTTG	Gubbels et al. 1999
<i>T. separata</i>	GGTCGTGGTTTTCTCGT	Schnittger et al. 2004
<i>T. taurotragi</i>	TCTTGGCACGTGGCTTTT	Gubbels et al. 1999
<i>T. velifera</i>	CCTATTCTCCTTTACGAGT	Gubbels et al. 1999
<i>Theileria</i> sp. (buffalo)	CAGACGGAGTTTACTTTGT	Oura et al. 2003
<i>Theileria</i> sp. (kudu)	CTGCATTGTTTCTTTCCTTTG	Nijhof et al. 2005
<i>Theileria</i> sp.(sable)	GCTGCATTGCCTTTTCTCC	Nijhof et al. 2005

^a Dr. Ard M. Nijhof (Institute of Parasitology and Tropical Veterinary Tropical Medicine, Free University, Berlin, Germany).

3.3.4. The *T. parva*-specific quantitative real-time PCR (qPCR)

A total of 159 DNA samples (119 from Botswana and 40 from Zimbabwe) were subjected to the *T. parva*-specific qPCR assay as previously described (Sibeko et al. 2008). Among the 120 smears collected from Botswana, one sample was discarded because of an insufficient amount of DNA for the test. The *T. parva*-specific forward (5' CTG CAT CGC TGT GTC CCT T 3') and *Theileria* genus-specific reverse (5' ACC AAC AAA ATA GAA CCA AAG TC 3') primers (Sibeko et al. 2008) were used to amplify a 167 bp fragment of the parasite V4 variable region of the 18S rRNA gene. For the specific detection of *T. parva* amplicons, a pair of hybridization probes: *T. parva* anchor (5' GGG TCT CTG CAT GTG GCT TAT-FL) and *T. parva* sensor (5' LCR640-TCG GAC GGA GTT CGC-PH) were included in the PCR reaction which consisted of 4 µl of 10x LightCycler-FastStart DNA Master^{PLUS} Hybridization Probes mix (with 2x final concentration), 0.5 mM of each primer, 0.1 mM of each hybridization probe, 0.5 U Uracil-deoxy-glycosylase (UDG) and 4 µl of the template DNA (30-50 ng) in a final volume of 20 µl. A *T. parva* positive buffalo DNA sample, 102 (Sibeko et al. 2008), and nuclease-free water were used as positive and negative controls, respectively. Amplification (a programme containing three steps, denaturing at 95°C for 10 sec,

primer annealing at 58°C for 10 sec, and product extension at 72°C for 15 sec, repeated for 45 cycles) and the melting curve analysis (samples were heated from 40°C up to 95°C with a heating rate of 0.2°C/sec) were done as previously described (Sibeko et al. 2008) in a LightCycler1 v2 (Roche Diagnostics, Mannheim, Germany). Fluorescence values were measured at 640 nm.

3.3.5. *The indirect fluorescent antibody test (IFAT)*

The IFAT was performed as described previously in other studies (Schindler and Mehlitz, 1969; Lohr and Ross 1969; Burrige, 1971). After discarding haemolysed samples, a total of 108 serum samples collected from the buffalo in Botswana were available for testing with the IFAT according to OIE standards (OIE manual, 2008). The test was conducted at the ARC-OVI using two dilutions, 1/40 and 1/80. The presence of fluorescence in both the 1/40 and 1/80 dilutions were considered as a positive result in serum from buffalo, indicative of the presence of *T. parva* antibodies (O. Mathee, personal communication).

3.3.6. *Statistical analysis*

Descriptive epidemiological measures were analyzed using Epi-Info software (CDC, Atlanta, USA) and were reported as percentages of positive animals to the different diagnostic tests. Chi square test calculations for homogeneity of two populations (Fischer exact test) were used to statistically evaluate the potential influence of age, sex, location and density of buffalo at the capture sites on the observed parasite prevalence. When the variance of the two groups was not homogenous, the Kruskal Wallis test was used. Values of $p < 0.05$ were considered significant. Agreement between the different diagnostic tests assessing the presence of *T. parva* or its antibodies (IFAT, qPCR and RLB) was calculated for those sera having a common result to those tests. Two by two comparisons of the results were expressed using the kappa value. Kappa is a widely used measure of test agreement defined as the quotient of the observed proportion of agreement beyond chance and the maximal proportion of agreement beyond chance (Cohen, 1960). A kappa of 0 indicates no agreement beyond chance, while a kappa of 1 indicates perfect agreement. A kappa of 0.5 indicates a moderate level of agreement. Only 107 samples from the IFAT could be used for the kappa test because of the absence of results for qPCR and RLB for one specific sample.

3.4. Results

3.4.1 *Detection of haemoparasites in buffalo blood samples using the RLB hybridization assay*

The RLB results (Table 2) indicated the presence of *Theileria*, *Babesia*, *Anaplasma* and *Ehrlichia* species, either as single or as mixed infections in the buffalo populations from two wildlife areas assessed in northern Botswana ($n = 120$) and one in Zimbabwe ($n = 40$).

Table 2: The occurrence of different haemoparasites in buffalo blood samples from two geographical areas, Chobe National Park and Okavango Delta, in northern Botswana and one from the Gonarezhou National Park in Zimbabwe as determined by the RLB hybridization assay.

	Chobe National Park (n=64)	Okavango Delta (n=56)	Northern Botswana total (n=120)	Gonarezhou National Park (n=40)	Overall total (n=160)
Single infections:	5 (7.8%)	18 (32.1%)	23 (19.2%)	9 (22.5%)	32 (20.0%)
<i>A. marginale</i>	1 (1.6%)	1 (1.8%)	2 (1.7%)	0	2 (1.3%)
<i>A. marginale</i> subsp. <i>centrale</i>	0	2 (3.6%)	2 (1.7%)	0	2 (1.3%)
<i>T. mutans</i>	3 (4.7%)	0	3 (2.5%)	2 (5.0%)	5 (3.1%)
<i>T. parva</i>	1 (1.6%)	15 (26.8%)	16 (13.3%)	3 (7.5%)	19 (11.9%)
<i>Theileria</i> sp. (buffalo)	0	0	0	1 (2.5%)	1 (0.6%)
<i>Theileria</i> sp. (sable)	0	0	0	3 (7.5%)	3 (1.9%)
Mixed infections	53 (82.8%)	27 (48.2%)	80 (66.7%)	26 (65.0%)	107 (66.9%)
<i>A. marginale</i>	13 (20.3%)	9 (16.1%)	22 (18.3%)	0	22 (13.8%)
<i>A. marginale</i> subsp. <i>centrale</i>	20 (31.3%)	14 (25.0%)	34 (28.3%)	10 (25.0%)	45 (28.1%)
<i>Anaplasma</i> sp. Omatjenne	1 (1.6%)	2 (3.6%)	3 (2.5%)	2 (5.0%)	5 (3.1%)
<i>B. bovis</i>	0	2 (3.6%)	2 (1.7%)	0	2 (1.3%)
<i>B. occultans</i>	26 (40.6%)	2 (3.6%)	28 (23.3%)	6 (15.0%)	34 (21.3%)
<i>E. ruminantium</i>	4 (6.3%)	3 (5.4%)	7 (5.8%)	0	7 (4.4%)
<i>T. bicornis</i>	0	0	0	5 (12.5%)	5 (3.1%)
<i>T. buffeli</i>	30 (46.9%)	4 (7.1%)	34 (28.3%)	6 (15.0%)	40 (25.0%)
<i>T. mutans</i>	36 (56.3%)	5 (8.9%)	41 (34.2%)	13 (32.0%)	54 (33.8%)
<i>T. ovis</i>	3 (4.7%)	1 (1.8%)	4 (3.3%)	0	4 (2.5%)
<i>T. parva</i>	32 (50.0%)	24 (42.9%)	56 (46.6%)	16 (40.0%)	72 (45.0%)
<i>T. taurotragi</i>	0	0	0	1 (2.5%)	1 (0.6%)
<i>T. velifera</i>	9 (14.1%)	1 (1.8%)	10 (8.3%)	7 (17.5%)	17 (10.6%)
<i>Theileria</i> sp. (buffalo)	8 (12.5%)	13 (23.2%)	21 (17.5%)	3 (7.5%)	24 (15.0%)
<i>Theileria</i> sp. (kudu)	0	0	0	1 (2.5%)	1 (0.6%)
<i>Theileria</i> sp. (sable)	25 (39.1%)	2 (3.6%)	27 (22.5%)	17 (42.5%)	44 (27.5%)
<i>Theileria/Babesia</i> genus-specific only	2 (3.1%)	5 (8.9%)	7 (5.8%)	3 (7.5%)	10 (6.3%)
<i>Anaplasma/Ehrlichia</i> genus-specific only	1 (1.6%)	3 (5.4%)	4 (3.3%)	0	4 (2.5%)
Negative/below detection limit	2 (3.1%)	1 (1.8%)	3 (2.5%)	6 (15.0%)	5 (3.1%)

3.4.1.1. Botswana

From a total of 120 blood smear samples tested, 23 samples (19.2%) contained single infections while 80 (66.7%) contained mixed infections. The most prevalent haemoparasite in the CNP was *T. mutans* (60.9%) followed by *T. parva* (51.6%), *T. buffeli* (46.9%), *B. occultans* (40.6%) and *A. marginale* subsp. *centrale* (31.3%). In the OD, *T. parva* (69.6%) was most prevalent followed by *A. marginale* subsp. *centrale* (28.6%) and *Theileria* sp. (buffalo) (23.2%) (Table 2; Figure 3 and 4). RLB results indicated that a total of 72 of the 120 samples (60.0%) tested positive for *T. parva* DNA.

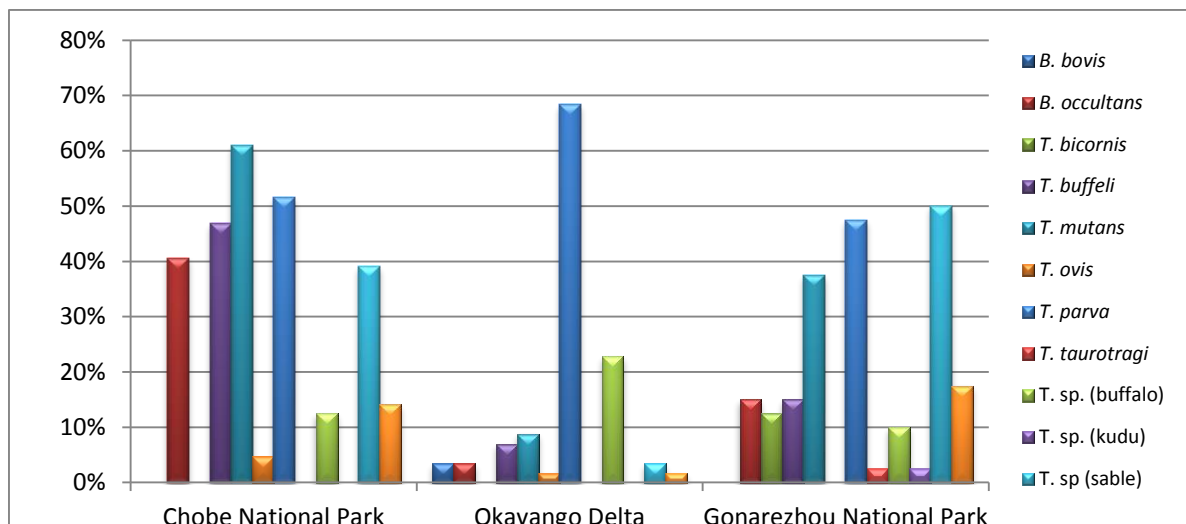


Figure 3: Graphical representation of the *Theileria* and *Babesia* species present in buffalo in the three study areas.

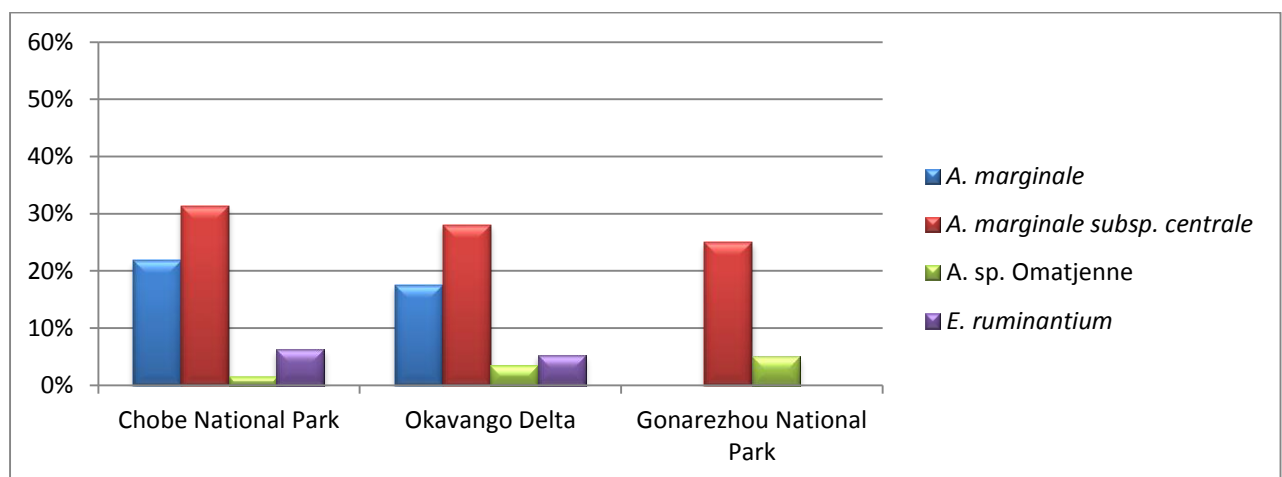


Figure 4: Graphical representation of the *Anaplasma* and *Ehrlichia* species present in buffalo in the three study areas.

RLB results showed significant differences in the prevalence of *T. mutans*, *T. buffeli*, *B. occultans*, *Theileria* sp. (sable) and *T. velifera* infections in the wildlife areas from northern Botswana (Table 3). There was a significant difference ($p \leq 0.05$) between the age of animals that harbored *A. marginale*

subsp. *centrale*, *T. buffeli*, *B. occultans* and *A. marginale* infections in the wildlife areas from northern Botswana (Table 3). There was a significant difference ($p \leq 0.05$) between sex of the buffalo that tested positive for *A. marginale* subsp. *centrale* and *T. velifera* DNA in northern Botswana (Table 3).

3.4.1.2. Zimbabwe

Single infections were identified in 9 (22.5%) of the samples, while mixed infections were identified in 26 (65.0%) samples in GNP. The most prevalent haemoparasite in the GNP was *Theileria* sp. (sable) (50.0%) followed by *T. parva* (47.5%), *T. mutans* (37.5%) and *A. marginale* subsp. *centrale* (25.0%) (Table 2; Figure 3 and 4). Only 15.0% of samples were found to be negative or below the detection limit of the test (Table 2).

RLB results indicated a total of 19 (47.5%) samples tested positive for *T. parva* DNA.

There was a significant difference ($p \leq 0.05$) in the prevalence of *B. occultans*, *T. bicornis* and *T. buffeli*, between CNP and GNP and for *T. mutans* and *Theileria* sp. (sable) between GNP and OD (Table 3). When comparing Botswana as a whole with Zimbabwe, significant differences ($p \leq 0.05$) were identified for *T. bicornis* and *Theileria* sp. (sable).

There were no significant differences between the prevalence of haemoparasites, other than *T. parva*, in the four different capture sites or the numbers were too small to assess statistical differences.

3.4.1.3. Detection of *T. parva* through RLB hybridization assay

Theileria parva DNA was detected in 33 (51.6%) and 39 (69.6%) buffalo samples from CNP and OD respectively, while 19 (47.5%) buffalo samples from GNP tested positive for *T. parva*. There was a significant difference ($p = 0.042$) in the prevalence of *T. parva*-positive samples determined by RLB between CNP and OD. However, there was no significant association between prevalence of *T. parva*, and sex or age of the sampled animals (Table 4). As a whole, there was a significant difference ($p \leq 0.05$) between CNP and OD and between OD and GNP.

When comparing the *T. parva* RLB results in the four buffalo capture sites (Figure 5), *T. parva* prevalence was highest in the NG30 samples (76.3%) followed by the MGR (55.6%) samples, CNP samples (51.6%) and GNP samples (47.5%). Differences in *T. parva* prevalence as determined by RLB were only significant when comparing NG30 versus CNP ($p \leq 0.05$) and NG30 versus GNP ($p \leq 0.05$) (Figure 5).

Table 3: Comparison of the occurrence of haemoparasites, other than *T. parva**, per wildlife area, sex and age as determined by the RLB hybridization assay. Significant differences ($p \leq 0.05$) between test results are indicated for CNP and OD (grey), CNP and GNP (red), OD and GNP (blue).

		<i>T. mutans</i>	<i>A. marginale</i> subsp. <i>centrale</i>	<i>T. buffeli</i>	<i>B. occultans</i>	<i>Theileria</i> sp. (sable)	<i>A. marginale</i>	<i>Theileria</i> sp. (buffalo)	<i>T. velifera</i>	<i>E. ruminantium</i>	<i>T. ovis</i>	<i>Anaplasma</i> sp. Omatjenne	<i>B. bovis</i>	<i>T. bicornis</i>	<i>Theileria</i> sp. (kudu)
Area	CNP	39/64 (60.9%)	20/64 (31.3%)	30/64 (46.9%)	26/64 (40.6%)	25/64 (39.1%)	14/64 (21.9%)	8/64 (12.5%)	9/64 (14.1%)	4/64 (6.3%)	3/64 (4.7%)	1/64 (1.6%)	0/64 (0.0%)	0/64 (0.0%)	0/64 (0.0%)
	OD	5/56 (8.9%)	16/56 (28.6%)	4/56 (7.1%)	2/56 (3.6%)	2/56 (3.6%)	10/56 (17.9%)	13/56 (23.2%)	1/56 (5.4%)	3/56 (5.4%)	1/56 (1.8%)	2/56 (3.6%)	2/56 (3.6%)	0/56 (0.0%)	0/56 (0.0%)
	GNP	15/40 (37.5%)	10/40 (25.0%)	6/40 (15.0%)	6/40 (15.0%)	20/40 (50.0%)	0/40 (0.0%)	4/40 (10.0%)	7/40 (17.5%)	0/40 (0.0%)	0/40 (0.0%)	2/40 (5.0%)	0/40 (0.0%)	5/40 (12.5%)	1/40 (2.5%)
Age	Young	16/38 (42.1%)	19/38 (50.0%)	6/38 (15.8%)	4/38 (10.5%)	7/38 (18.4%)	15/38 (39.5%)	4/38 (10.5%)	2/38 (5.3%)	2/38 (5.3%)	2/38 (5.3%)	0/38 (0.0%)	1/38 (2.6%)	0/38 (0.0%)	0/38 (0.0%)
	Adult	27/77 (35.1%)	16/77 (20.8%)	27/77 (35.1%)	24/77 (31.2%)	20/77 (26.0%)	8/77 (10.4%)	15/77 (19.5%)	8/77 (10.4%)	5/77 (6.5%)	2/77 (2.6%)	3/77 (3.9%)	0/77 (0.0%)	0/77 (0.0%)	0/77 (0.0%)
Sex	Male	21/45 (46.7%)	19/45 (42.2%)	16/45 (35.6%)	14/45 (31.1%)	13/45 (28.9%)	13/45 (28.9%)	10/45 (22.2%)	7/45 (15.6%)	4/45 (8.9%)	3/45 (6.7%)	1/45 (2.2%)	1/45 (2.2%)	0/45 (0.0%)	0/45 (0.0%)
	Female	22/70 (31.4%)	16/70 (22.9%)	17/70 (24.3%)	14/70 (20.0%)	14/70 (20.0%)	10/70 (14.3%)	9/70 (12.9%)	3/70 (4.3%)	3/70 (4.3%)	1/70 (1.4%)	2/70 (2.9%)	0/70 (0.0%)	0/70 (0.0%)	0/70 (0.0%)

* For details of *T. parva* results, refer to Table 4

Table 4: Comparison of prevalence of *T. parva* per location, age and sex with the three different tests performed. Significant differences ($p \leq 0.05$) between test results are indicated for CNP and OD (grey), OD and GNP (blue)

Parameter		RLB	IFAT	qPCR
Wildlife Area	CNP	33/64 (51.6%)	39/60 (65.0%)	49/63 (77.8%)
	OD	39/56 (69.6%)	41/48 (85.4%)	47/56 (83.9%)
	GNP	19/40 (47.5%)	-	28/40 (70.0%)
Age	Young	19/38 (50.0%)	26/36 (72.2%)	27/38 (71.1%)
	Adult	48/77 (62.3%)	54/71 (76.1%)	66/77 (85.7%)
Sex	Male	23/45 (51.1%)	31/43 (72.1%)	37/45 (82.2%)
	Female	44/70 (62.9%)	49/64 (76.6%)	57/70 (81.4%)

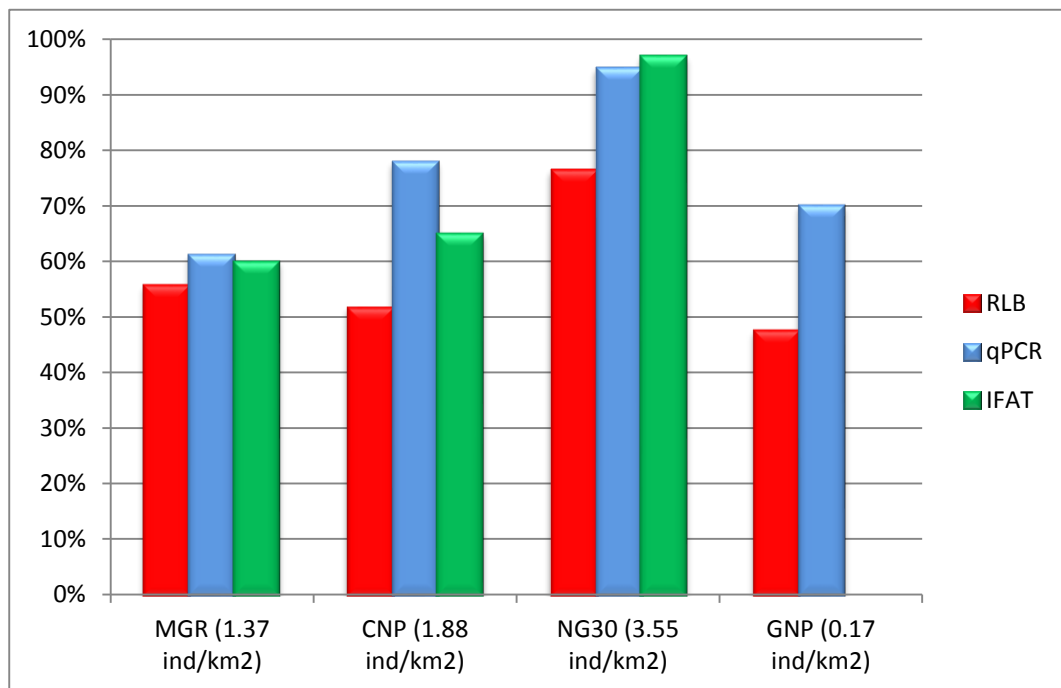


Figure 5: Comparison of capture location and prevalence of *T. parva* according to the different tests performed. Differences were significant ($p \leq 0.05$) when comparing CNP and NG30 (for the 3 tests), MGR and NG30 (for IFAT and qPCR) and for GNP and NG30 (RLB and qPCR).

3.4.2. IFAT

T. parva antibodies were detected in 80 of the 108 (74.1%) Botswana samples tested (Table 4). Of these, 40 (37.0%) samples tested positive at 1/80 and 40 (37.0%) at 1/40 dilutions. More buffalo from

OD (85.4%) than from CNP (65.0%) were seropositive for *T. parva* (Table 4) and this difference was significant ($p= 0.016$). There was no significant difference between the age and sex of the animals that were seropositive (Table 4).

The mean seroprevalence was higher (96.9%) in the NG30 region than in the other two capture sites, 65.0% in the CNP and 60.0% in the MGR and those differences were highly significant (Figure 5).

3.4.3. Quantitative real-time PCR (qPCR)

A total of 159 DNA samples (119 from Botswana and 40 from Zimbabwe) were subjected to the *T. parva*-specific qPCR assay. Melting curve analysis (Figure 6) confirmed the presence of *T. parva* DNA in 96 of 119 (80.7%) Botswana samples and 28 of 40 (70.0%) Zimbabwe samples tested. There were no significant differences between prevalence of *T. parva* with this test, when comparing ages and sex of the animals between CNP and OD (Table 4).

Mean prevalence of *T. parva* as determined by qPCR was higher in the NG30 region (94.7%) than in the other three capture sites, 77.8% in the CNP, 70.0% in GNP and 61.1% in the MGR, respectively (Figure 5). The differences in *T. parva* prevalence between MGR and the CNP were not significant but it became highly significant when comparing *T. parva* prevalence in NG30 with that in the other two areas in Botswana and in GNP, Zimbabwe (Figure 5).

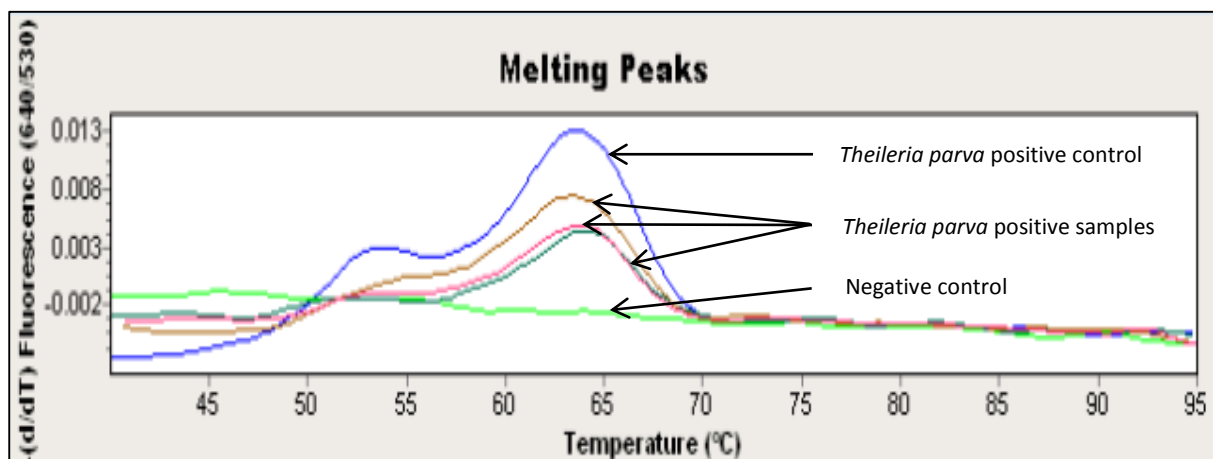


Figure 6: Graphical representation of real-time PCR melting peak results. Representative melting curves at $\pm 63^{\circ}\text{C}$ at 640 nm are shown, which confirm the presence of *T. parva* positive samples.

3.4.4. Comparison of tests

For IFAT, 80/108 (74.1%) samples tested positive for *T. parva* antibodies, while for the qPCR and the RLB hybridization assay, 96/119 (80.7%) and 72/120 (60.0%) of the samples tested positive in Botswana, respectively compared to 28/40 (70.0%) for qPCR and 19/40 (47.5%) for RLB in Zimbabwe. The Kappa value when comparing IFAT and qPCR indicated a moderate level of

agreement (0.561), while the comparison with RLB and the two other tests indicated a low level of agreement in Botswana. A low level of agreement was also observed for qPCR and RLB in Zimbabwe (Table 5).

Table 5: Agreement expressed by kappa value when comparing diagnostic tests for *Theileria parva* two by two in Botswana and Zimbabwe. Grey shading indicates a significant difference between test results for a given parameter ($p \leq 0.05$).

Tests compared	Sample size	Kappa value	Standard error 95% CI
<u>Botswana</u>			
RLB vs qPCR	119	0.256	0.09 [0.09 ; 0.472]
IFAT vs qPCR	107	0.561	0.096 [0.3 ; 0.7]
IFAT vs RLB	107	0.154	0.094 [-0.031 ; 0.3]
<u>Zimbabwe</u>			
RLB vs qPCR	40	0.265	0.136 [-0.001 ; 0.531]

3.5. Discussion

This is the first report on the presence of tick-borne haemoparasites in African buffalo from two of the most representative wildlife areas in northern Botswana and one in Zimbabwe. Our results provide new insights on the distribution of haemoparasites transmissible to cattle across the two main wildlife areas in northern Botswana and the one in Zimbabwe which can be inferred to the distribution of their specific vectors. Several important pathogenic haemoparasites were identified which could present a constraint to the livestock industry in these areas. These included *T. parva*, *A. marginale*, *B. bovis* and *E. ruminantium*. More mixed infections of haemoparasites were found in buffalo from CNP than OD and GNP. The higher level of mixed infections in CNP may be due to buffalo coinciding with the tick vectors in their habitats or the closer clustering of buffalo herds making transmission of the parasites easier. Indeed, large herds of buffalo congregate along the Chobe river during the dry season which might facilitate the exchange of ticks and their parasites between different individuals.

The RLB, IFAT and qPCR tests all indicated a high prevalence of *T. parva* in the study areas. This indicates a high risk of spreading Corridor disease caused by *T. parva* from buffalo to cattle by the vector ticks at the wildlife-livestock interface. Other haemoparasites with high prevalence identified by the RLB included: *T. mutans*, *T. buffeli*, *B. occultans* and *Theileria* sp. (sable) in CNP, *A. marginale* subsp. *centrale*, *Theileria* sp. (buffalo) and *A. marginale* in OD and *T. mutans*, *T. parva* and *Theileria* sp. (sable) in GNP. Generally speaking, the buffalo population in the OD sample had

lower levels of haemoparasite infection than the buffalo in the CNP and GNP, with the exception of *Theileria* sp. (buffalo) and to a lesser extent *Anaplasma* sp. Omatjenne and *B. bovis* (for the latter two parasites, very few positives were detected in any of the parks). In the specific case of *T. parva*, a significant association was observed between densities of buffalo in the capture location of the herds (although admittedly only three measures of density were available) and prevalence found with the different tests, particularly in the case of the IFAT and qPCR results. This was particularly true between MGR and the NG30 where differences between density figures were more extreme. What should be added is that the densities used were general approximations of the parks. Also, the densities of the different study areas were not measured with the same methods but are likely to be significantly different.

However, buffalo density is only one possible cause of those differences, and many other habitat variations or ecological factors affecting host health or vector distribution and density in the different range areas of the buffalo herds sampled could also be responsible for those parasite differences (Tompkins et al., 2011; Anderson et al., 2013). Therefore, further studies with more data and measurements at the different herd locations would be necessary to detect explanatory factors accounting for those prevalence differences.

When comparing Botswana as a whole to Zimbabwe, results indicated the following parasites identified to be similar between the countries: *Anaplasma* sp. Omatjenne, *A. marginale* subsp. *centrale*, *B. occultans*, *T. buffeli*, *T. mutans*, *T. parva*, *Theileria* sp. (buffalo), *Theileria* sp. (sable) and *T. velifera*. Interestingly, *A. marginale* and *E. ruminantium* were not identified in samples from Zimbabwe or they were simply at too low a level to be detected. *Theileria bicornis* and *Theileria* sp. (kudu) were solely identified in Zimbabwe and were both peculiar finds in buffalo. Significant differences between the countries were found when comparing *A. marginale*, *T. bicornis* and *Theileria* sp. (sable).

T. parva antibodies were detected in 74.1% of samples tested using the IFAT in Botswana. Limitations of the IFAT include standardization of the test in buffalo samples, subjectivity towards the interpretation of results acquired and the difficulty of detecting low levels of parasite antibodies (Norval et al., 1992; BurrIDGE and Kimber, 1972). The IFAT is highly sensitive when testing for antibodies for only one species of *Theileria*, but in areas where different species overlap; cross-reactions between *Theileria* species are common (especially between *T. parva*, *T. annulata* and *T. taurotragi*) and reduce the specificity of the test (Norval et al., 1992; OIE Manual, 2008). However, the geographical distribution of *T. annulata* and *T. parva* does not overlap (Fujisaki et al., 1994) and *T. taurotragi* was not identified in the Botswana buffalo samples. Therefore, the likelihood of cross-reactivity can be ruled out and our results most likely reflect the real presence and burden of *T. parva*,

which would explain the high level of agreement between IFAT and qPCR. Another factor to take into account is the period elapsed from infection and the development of antibodies. With the use of schizont antigen, *T. parva* antibodies can first be detected 10 to 14 days post-infection and with piroplasm antigens 15 to 21 days post-infection. High levels of antibodies are still detectable 30 to 60 days after the animals have recovered from a *T. parva* infection which is followed by the gradual decrease of antibody levels. Antibodies can still be detected 4 to 6 months post-recovery and may persist for up to a year at such low levels that they may not be detected at a serum dilution of 1/40 (OIE Manual, 2008). The animals in our study may have carried *T. parva* infections for a very long time as suggested by the very low antibody levels observed.

Comparing the efficiency of the different tests in northern Botswana, we found that the qPCR (81.4%) and IFAT (74.1%) were far better in identifying *T. parva* positive samples than the RLB assay (60.0%). When comparing the *T. parva*-specific tests done for Zimbabwe samples it was found that qPCR (70.0%) was more effective than the RLB (47.5%). Weak hybridization and cross-reactivity of probes may cause the RLB hybridization assay to yield less sensitive results than the qPCR and IFAT (O'Sullivan et al., 2011).

Risk factors influencing the prevalence of tick-borne parasites may include the distribution of tick vectors, the abundance of buffalo and cattle and their movement/migratory patterns, resistance of the hosts to the parasites and their tick vectors (Bakheit and Latif, 2002) and age of the host (Anderson et al., 2013). It has previously been found that older animals have a higher tick load than younger ones (Anderson et al., 2013). However, higher tick loads do not necessarily mean higher infection rates of haemoparasites. A model designed in East Africa also determined that at wildlife-livestock interfaces where only the cattle are treated with acaricides, *T. parva* remains a problem because this treatment has no effect on the disease transmission in buffalo. In addition, the continuous use of acaricides can have significant economic and ecological consequences (Walker et al., 2014).

Our study identified significant differences in parasite infections between buffalo of different ages in northern Botswana: higher infection in young (*A. marginale*, *A. marginale* subsp. *centrale*) and in adult animals (*B. occultans* and *Theileria buffeli*). Significant differences between infection rates in the three study areas were identified for some of the parasites: *T. mutans*, *T. buffeli*, *B. occultans*, *Theileria* sp. (sable) and *A. marginale* but only *T. velifera* and *T. bicornis* when comparing two of the areas. Significant differences between the sex of the animals were identified in the two areas of northern Botswana for some of the parasites: *A. marginale* subsp. *centrale* and *T. velifera*. However, it is likely that our sample was too small to be able to detect a consistent trend between age and infection.

A number of studies have previously been conducted on buffalo in South Africa. Pienaar et al. (2011) tested buffalo in the Marakele National Park (MNP) and Kruger National Park (KNP) for the presence of *Theileria* spp. using the RLB hybridization assay and the *T. parva*-specific qPCR assay. The RLB results indicated the presence of *T. parva*, *Theileria* sp. (buffalo), *T. mutans*, *T. buffeli* and *T. velifera* in both parks. The qPCR assay identified 70% of samples positive for *T. parva* and the RLB results indicated 40% of samples. In a separate study by Chaisi et al. (2011) in the Hluhluwe-iMfolozi Park, Greater Limpopo Transfrontier Park and Kruger National Park, *T. parva*, *Theileria* sp. (buffalo), *T. mutans*, *T. buffeli* and *T. velifera* were also identified in African buffalo. In both studies, the *T. parva*-specific qPCR was found to be more sensitive than the RLB, correlating with the results found in our study. A study done by Debeila (2011) on buffalo in the Hluhluwe-iMfolozi Park and Kruger National Park found the same *Theileria* spp. as our study in Botswana, as well as *A. marginale* subsp. *centrale*, *A. marginale*, *Anaplasma* sp. Omatjenne, *E. ruminantium* and *B. occultans*. This serves as another report of *Anaplasma* sp. Omatjenne being identified in buffalo, Debeila (2011) identified 16.5% to be positive in their samples, but the parasite was found in very low levels (3.1%) in our study. *B. occultans* causes a benign form of cattle babesiosis and was also reported in South Africa by Debeila (2011) for the first time. Our study identified 21.1% samples to be positive compared to the study in Hluhluwe-iMfolozi Park, South Africa (50.0%). This parasite was first obtained on a farm in the Limpopo province, South Africa in 1976 (Thomas and Mason, 1981).

In a study done by Oura et al. (2011), it was shown that buffalo from four national parks in Uganda were carriers of *T. parva*, *T. mutans*, *T. velifera*, *A. marginale* and *A. marginale* subsp. *centrale*. In two of these parks, the buffalo also carried *T. buffeli* and *Theileria* sp. (buffalo). None of the animals sampled were carriers of *T. taurotragi*, *B. bovis*, *B. bigemina*, *A. bovis* or *E. ruminantium*. As in Uganda, the pathogenic *B. bovis* has previously been reported to be absent from buffalo in Botswana (Penzhorn, 2006) but present in cattle (Sharma et al. 2000). However, in the current study we identified the parasite to be present in a low percentage of the OD buffalo but none in those from CNP. Although *B. bovis* has been reported in cattle from Zimbabwe (Smeenk et al. 2000), none of the buffalo samples from GNP were positive. *E. ruminantium* could be identified in a few CNP and OD buffalo tested. The significance of buffalo as possible reservoir host of some of these economically important haemoparasites (i.e. *A. marginale*, *E. ruminantium*) remains unknown.

Theileria sp. (sable), which is fatal to sable (*Hippotragus niger*) and roan antelope (*Hippotragus equinus*), but non-pathogenic to buffalo (Nijhof et al. 2005) was identified in some of the Botswana buffalo. However, it should be noted that the positive RLB signals might be due to cross reactions of the *Theileria* sp. (sable) probe with genotypes similar to *Theileria* sp. (sable) and/or with *T. velifera* and should be interpreted with caution (Mans et al. 2011). Similarly, four samples tested positive for *T. ovis* which is usually found in goats and sheep. We can only speculate whether these are true

findings due to incidental infections, or whether they are as a result of cross-reaction of the RLB probes with previously unknown targets or contamination with other target DNA.

The following important tick vectors have been identified in Botswana in previous studies: *Amblyomma variegatum*, *Rhipicephalus decoloratus*, *R. zambeziensis*, *R. evertsi evertsi*, *R. simus*, *Hyalomma truncatum* and *H. marginatum rufipes* (Musuka et al., 2001; Paine, 1982; Walker et al., 1978). Important ticks identified previously in Zimbabwe include: *Amblyomma hebraeum*, *A. variegatum*, *Rhipicephalus appendiculatus*, *R. decoloratus*, *R. evertsi evertsi*, *R. microplus*, *R. simus*, *R. zambeziensis*, *Hyalomma truncatum* and *H. marginatum rufipes* (Norval, 1980; Hove et al. 2008). These ticks are known to transmit most of the haemoparasites found in this study. These vectors may also be responsible for the transmission of *T. buffeli* and *Theileria* sp. (buffalo) but further analysis is needed to confirm this hypothesis, since the tick vectors of these parasite species are still unknown.

T. parva is known to occur in South Africa (Anonymous, 1981; Neitz, 1955), Zambia (Munang'andu et al., 2009) and Zimbabwe (Latif and Hove, 2011; Caron et al. 2013), but this is the first report of its presence in Botswana, despite its presence being suspected for several years already. Currently, there are no regulations instituted for the systematic surveillance and control of tick-borne diseases in Botswana. In the 1980s ticks and tick-borne diseases were regulated through compulsory dipping of cattle on a weekly basis in Zimbabwe (Thompson, 1984). But in the mid 1990s, the country moved over to the use of vaccines in problem herds and a shift from intensive dipping to a more reduced regime which would establish enzootic stability in the country (Morzaria and Williamson, 1997). The last documented disease surveillance in Zimbabwe occurred in the early 1990s (Caron et al. 2013). In addition, Corridor disease in cattle is fulminant and makes it difficult to detect clinical cases in live animals. Therefore, the present work emphasizes the role of the African buffalo, as a sentinel species to identify the presence and circulation of livestock pathogens. The presence of *Rhipicephalus appendiculatus* and East Coast fever in northern Botswana has been predicted by spatial risk models by some authors (Olwoch et al., 2008). However, this is the first confirmation of the presence of *T. parva* in buffalo in northern Botswana which act as asymptomatic reservoirs of these haemoparasites. When infected buffalo share the same home ranges as cattle, the haemoparasites can be transmitted to cattle through infected tick bites. This information on the circulation of TBD can contribute to raise awareness among veterinary officials and cattle owners so that control measures (prevention of wildlife-cattle contacts, regular dipping) can be implemented to mitigate their economic impact. In a recent comparative assessment of cattle herds in three different wildlife/livestock interfaces in Zimbabwe, significantly higher levels of *T. parva* antibodies were found in those areas that were unfenced when compared with those that had a physical separation between wildlife and livestock (Caron et al., 2013). Therefore, we can hypothesize that this parasite is less likely to be transmitted from buffalo to cattle in the OD, due to the presence of a veterinary cordon fence preventing contacts

with cattle surrounding the game reserve. To the contrary in the CNP, where there is no physical separation between buffalo and cattle, transmission of common diseases from buffalo to cattle is likely to occur more frequently (Jori et al., 2013) and future surveillance efforts should be targeted in priority towards livestock from these areas

3.6. Conclusion

This study illustrates the diversity of haemoparasites present in African buffalo from northern Botswana and Zimbabwe and highlights the role of African buffalo as a sentinel species for tick-borne pathogens of livestock. Our results indicate the significance of the African buffalo as reservoir host for important tick-borne haemoparasites that can cause severe disease in cattle. Important tick-borne haemoparasites identified in this study included: *T. parva*, *A. marginale*, *B. bovis* and *E. ruminantium*. Haemoparasites other than *T. parva* are unlikely to pose a significant risk livestock. They can in fact contribute to endemic stability to these tick-borne diseases. However, *T. parva* is of great significance, as it is difficult or impossible, to achieve endemic stability in a cattle population due to the genetic diversity, especially where buffalo occur. The virulence of the parasite and apparent lack of an age-associated innate resistance, as well as the complexity and cost of producing vaccines, further contribute to the economic importance. Our results also suggest that qPCR and IFAT are more efficient in detecting buffalo exposed to *T. parva* than the RLB test. This study should contribute to raise awareness among veterinary authorities regarding the potential occurrence of these parasites in cattle so that appropriate control and surveillance protocols, taking into account the presence of infected wildlife reservoirs in those areas, can be designed at the wildlife-livestock interface.

3.7. References

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Chapter 4

Characterization of *Theileria parva* in African buffalo (*Syncerus caffer*) from Botswana and Zimbabwe using the p67, p104 and polymorphic immunodominant molecule (PIM) antigenic genes

4.1. Abstract

Bovine theileriosis, caused by *Theileria parva*, has had severe implications to the cattle industry. There is little information on the distribution of this disease and its vector ticks in northern Botswana and Zimbabwe. The African buffalo (*Syncerus caffer*) is considered to be the reservoir host for the parasite. Several *T. parva* antigen genes have been identified as good candidates to use for the differentiation between buffalo-derived and cattle-derived *T. parva* isolates. These include p67, p104 and the polymorphic immunodominant molecule (PIM). An attempt was made to characterize *T. parva* through the size differentiation of p67 genotypes and characterization of the variable regions of the *T. parva* antigen genes, p104 and PIM, by using semi-nested PCR-RFLP profiles. These p104 and PIM profiles were also compared to sample profiles collected from different areas in South Africa to establish the genetic diversity of the *T. parva* isolates amongst the three countries. The p67 gene could only be amplified in 13 (14%) and 9 (32%) *T. parva*-positive samples from Botswana and Zimbabwe, respectively, but three of the four known p67 alleles were identified; the 1.1 kb allele being the most abundant. For the p104 gene, 82 (85%) and 13 (46%) *T. parva*-positive samples from Botswana and Zimbabwe, respectively, could be amplified and 56 (58%) and 5 (18%) *T. parva*-positive samples from Botswana and Zimbabwe, respectively, for PIM. Cluster analysis for p104 showed that samples from Botswana and Zimbabwe clustered together with samples from Hluhluwe-iMfolozi (South Africa). The cluster analysis of PIM revealed that the Botswana and Zimbabwe samples mostly clustered separately from those from South Africa. The complexity of p67 genotypes was reconfirmed and could not be used to differentiate between buffalo-derived and cattle-derived *T. parva* isolates. The complexity of the *T. parva* gene diversity observed may be attributed to the high number of buffalo translocations in southern Africa. Sequencing data is required to better understand the significance of these genotypes in the epidemiology of theileriosis in Botswana and Zimbabwe. Mixed infections of p104 and PIM profiles proved to be too complex to successfully determine known profiles and further analysis of cloned single infections of these genes as well as sequencing data is required.

4.2. Introduction

Theileria parva, a tick-borne protozoan parasite, is the causative agent of East Coast fever (ECF), Corridor disease and January disease in cattle and buffalo (Theiler, 1904; Neitz, 1955; Lawrence, 1992). Diseases caused by *T. parva* are of major economic importance in central, eastern and southern Africa (Young et al. 1988). The African buffalo (*Syncerus caffer*) plays an important role as natural reservoir host from which the parasite is transmitted by *Rhipicephalus appendiculatus*, *R. zambeziensis* and *R. duttoni* (Lawrence et al. 1983, Uilenberg, 1999).

East Coast fever, which is an endemic cattle disease in eastern Africa, was introduced into southern Africa in the early 1900s through cattle importation from East Africa. The disease was later eradicated after an intensive campaign which included movement control, tick control, destocking of infected pastures and slaughter (Anonymous, 1981). It was finally eradicated from southern Mozambique by 1917, from Zimbabwe by 1954, from South Africa by 1955 and Swaziland by 1960. After the eradication of ECF in Zimbabwe, another form of theileriosis, known as January disease, emerged. The causative agent was named *Theileria bovis* (Lawrence, 1979; Uilenberg et al., 1982). In South Africa, Corridor disease was first diagnosed in 1953 after infected buffalo came into contact with naïve cattle on a corridor of land between the Hluhluwe and iMfolozi game reserves (Neitz, 1955). The clinical signs and pathology of the disease resembled ECF, but no piroplasms were seen in blood smears of reacting cattle. Corridor disease was thus considered to be caused by a different parasite, which was named *Theileria lawrencei* (Neitz, 1955). The parasites causing ECF, Corridor Disease and January disease are morphologically and serologically indistinguishable (Lawrence, 1979; Burridge et al., 1974), but based on the clinical and epidemiological features of the diseases they caused, they were originally regarded as three distinct species or subspecies of *T. parva*. This trinomial naming system has since been discarded due to a lack of biological evidence for discrimination of the subspecies, and the *T. parva* parasites are now referred to as cattle-derived or buffalo-derived according to their host of origin (Perry & Young, 1993). Thus, ECF and January disease result from cattle-to-cattle transmission of cattle-derived *T. parva* while Corridor disease results from buffalo-to-cattle transmission of buffalo-derived *T. parva* (Neitz, 1957; Koch et al., 1988).

Several genes have been investigated which could be used to differentiate between cattle- and buffalo-derived *T. parva* isolates, including the antigenic genes p67, p104, p150 and the polymorphic immunodominant molecule (PIM). The p67 gene codes for a sporozoite antigen and plays a role in the parasite entry into host lymphocytes (Iams et al. 1990; Nene et al. 1996; Toye et al. 2014). Research in East Africa has shown the presence of a 129 bp deletion in the central region of the p67 gene in cattle-derived isolates (designated allele 1 by Sibeko et al., 2010), while buffalo-derived isolates had no deletion (designated allele 2 by Sibeko et al., 2010) (Nene et al., 1992; Nene et al., 1996). It was,

therefore, assumed that the presence or absence of this deletion could be used for characterization (Nene et al., 1996). The p67 genotypes could, however, not be successfully used in South Africa to differentiate between cattle- and buffalo-derived *T. parva* parasites. This study revealed the presence of four p67 genotypes, including those previously found in *T. parva* isolates from East Africa, as well as two novel genotypes; one with a different 174 bp deletion (allele 3) and the other with a similar sequence to allele 3 but with no deletion (allele 4) (Sibeko et al., 2010).

The p104 protein codes for a 104 kDa rhoptry antigen and is expressed by the sporozoite stage of *T. parva*; it is capable of inducing sporozoite-neutralizing antibodies (Iams et al., 1990). PIM is believed to play a role as target antigen in the induction of cytotoxic T cell responses (Shapiro et al. 1987; Toye et al. 1991; Shaw, 2003) and is expressed by both the sporozoite and the schizont stages of the parasite. PCR-RFLP profiles of the variable regions of both the p104 and PIM genes have previously been used to differentiate between buffalo- and cattle-derived *T. parva* isolates on the basis of the level of polymorphism, with less polymorphic profiles being associated with cattle-derived *T. parva* parasites and more polymorphic profiles with buffalo-derived parasites (Geysen et al., 1999; Geysen, 2000; Bishop et al., 2001; De Deken et al., 2007). In the study by Sibeko et al. (2011), it was shown that p104 and PIM PCR-RFLP profiles are more complex than previously thought and the differentiation of buffalo- and cattle-derived *T. parva* parasites in South Africa based on these profiles was not be possible.

The aim of this study was to investigate whether size differentiation of the central region of the p67 *T. parva* antigen gene and semi-nested PCR-RFLP profiles of the variable regions of the p104 and PIM genes could be used to differentiate cattle-type *T. parva* from buffalo-derived isolates circulating in two distinct buffalo populations from northern Botswana and one population from southern Zimbabwe. The results obtained were also compared to those previously found by Sibeko et al. (2011) in buffalo from South Africa.

4.3. Materials and Methods

4.3.1. Sample collection

A total of 96 *T. parva* positive buffalo samples from two locations in northern Botswana, namely the Chobe National Park (CNP) (n=49) and Okavango Delta (OD) (n=47) were characterised, as well as 28 *T. parva* positive buffalo samples from the Gonarezhou National Park (GNP) in the south-eastern part of Zimbabwe. *Theileria parva* positive samples were selected using a *T. parva*-specific real-time PCR assay (Sibeko et al., 2008). Buffalo blood from Botswana was preserved on Giemsa-stained slides, while buffalo blood from Zimbabwe was preserved on FTA filter paper. (See Chapter 3 for details and maps).

4.3.2. DNA extraction

Genomic DNA was extracted from Giemsa-stained slides and FTA filter paper using the QIAmp DNA mini kit (Qiagen) following the manufacturer's protocol. The DNA for all samples were eluted into 100 µl of TE buffer and stored at –20°C until further use.

4.3.3. Nested PCR amplification and size differentiation of the p67 gene from *T. parva*

Forward primer 613 (5'-ACA AAC ACA ATC CCA AGT TC-3') and reverse primer 792 (5'-CCT TTA CTA CGT TGG CG-3') (Nene et al., 1996) were used to amplify the variable region of the p67 gene. The High Fidelity PCR Master System (Roche Diagnostics, Mannheim, Germany) was used to prepare PCRs according to Nene *et al.* (1996). Each reaction contained 5 µl DNA (30-50 ng), 0.1 µM of each primer, 1.5 mM MgCl₂, 200 µM dNTPs, High Fidelity Enzyme blend (concentration unknown) and nuclease-free water to a total volume of 25 µl. The cattle-derived *T. parva* Muguga stock from Kenya which is responsible for ECF (Brocklesby et al., 1961), and nuclease-free water were used as positive and negative controls, respectively. Amplification was performed in a Perkin-Elmer 9600 thermocycler (Applied Biosystems, South Africa) using 40 cycles comprising of 95°C for 1 min, 55°C for 1 min and 74°C for 1 min (Nene *et al.* 1996). For the secondary PCR, 0.5 µl of the primary PCR product was used as a template; the same protocol was followed as above except the amplification cycles were reduced from 40 to 25. The PCR products were visualized on a 2% ethidium bromide-stained agarose gel.

4.3.4. PCR-RFLP analysis of the p104 gene from *T. parva*

The PCR for the amplification of the *T. parva* p104 antigenic gene was prepared as follows: A volume of 5 µl DNA (30-50 ng) was used in a 50 µl reaction mix containing DreamTaq Green PCR master mix (2x) (Thermo scientific, United States of America) [DreamTaq DNA polymerase, 2X DreamTaq Green buffer, dATP, dCTP, dGTP and dTTP, 0.4 mM each, and 4 mM MgCl₂], 0.5 µM of the forward primer p104F2 (5'-CCA CCA TCT CCT AAA CCA CCG TT-3') and 0.5 µM of reverse primer p104R (5'-TAA GAT GCC GAC TAT TAA TGA CAC CAC AA-3') (De Deken *et al.*, 2007) and made up to the final volume using nuclease-free water according to the manufacturer's instructions. The cattle-derived *T. parva* Muguga stock from Kenya (Brocklesby et al., 1961) and known cattle-derived *T. parva* stock (Katete) (Geysen et al. 2000) were used as positive controls and nuclease-free water as the negative control. The PCR program consisted of 30 cycles of 95°C for 1 min, 58°C for 1 min and 74°C for 1 min (De Deken *et al.* 2007), using the Perkin-Elmer 9600 thermocycler (Applied Biosystems, South Africa). For the secondary PCR, 0.5 µl of the primary PCR product was used as template. The same reaction and cycling conditions were used as described above, except forward primer p104nF (5'-AAC CAC CGT TTG ATC CAT CAT TCA-3') and an annealing temperature of 60°C were used.

Amplicons were subsequently digested with the restriction enzyme *AluI* (5'...AG CT...3') as previously described by De Deken *et al.* (2007) and incubated at 37°C for 8 hours where after the fragments were analyzed on a 2% high resolution agarose gel (Sigma Aldrich) which ran for 40 min at 100 volts. Bands were visualized under a UV light.

4.3.5. PCR-RFLP analysis of the PIM gene from *T. parva*

The *T. parva* PIM antigenic gene was amplified using primers PIM forward 1 (5'-GTG AAT GTT GTG ATC TTA ATC C-3') and PIM reverse R4 (5'-CCC ACA ACC GTG GAA TGG CGT A-3') (De Deken *et al.*, 2007). The PCR mixture was prepared as follow: A volume of 5 µl DNA (30-50 ng) was used in a 25 µl reaction mix containing 2x MyTaq HS PCR reaction mix (Bioline, United Kingdom) [MyTaq buffer, dNTPs, MgCl₂, enhancers and stabilizers, concentrations unknown], 0.5 µM of each primer, made up to the final volume using nuclease-free water. Two *T. parva* cattle-derived DNA samples, Muguga (Brocklesby *et al.*, 1961) and Katete (Geysen *et al.* 2000) were used as positive controls and nuclease-free water as thenegative controls. The Perkin-Elmer 9600 thermocycler (Applied Biosystems, South Africa) was used and the PCR programme consisted of 30 cycles of 95°C for 1 min, 58°C for 1 min and 74°C for 1 min (De Deken *et al.* 2007). The secondary PCR used 0.5 µl of the primary PCR product as template, primers Fm (5'-ATT CCA CTG GTT CTT CCG ATS TA-3') and reverse R4 were used with the same cycling conditions described above, except an annealing temperature of 62°C was used.

The PCR amplicons were digested with the *BclI* restriction enzyme (5'...T GATCA...3') as previously described in De Deken *et al.* (2007) and incubated at 50°C for 8 hours; the digested products were separated on a 2% high resolution agarose gel (Sigma Aldrich) which ran for 40 minutes at 100 volts. Bands were visualized under a UV light.

4.3.6. PCR-RFLP profile analysis

The p104 and PIM PCR-RFLP profiles obtained were analyzed using the Bionumerics software package 5.1 (Applied Maths, Kortrijk, Belgium). Known cattle-derived *T. parva* stock (Muguga, Katete) and buffalo-derived *T. parva* (KNP102) were included in the analysis as reference profiles. Buffalo and cattle *T. parva* p104 and PIM PCR-RFLP profiles, previously generated by Sibeko *et al.* (2009), were also included (Table 4.1). Normalization was done using the molecular weight marker 100 bp DNA ladder (Fermentas Life Sciences, Germany), which was run in two lanes per gel per 15 lane gel. DNA fragments of less than 100 bp were excluded from the analysis as their size could not be estimated accurately using the 100 bp DNA ladder; or in some instances these fragments had run off the gel. The software was used to calculate Dice coefficients of similarity, to cluster the RFLP profiles and to generate dendrograms by the unweighted-pair group method using average linkages

(UPGMA). The most appropriate settings for optimization and tolerance, as determined by the software, were calculated.

Table 4.1: Geographic location and source of blood samples used for p104 and PIM PCR-RFLP characterization of *T. parva* parasites.

Geographical location and animal of origin of blood sample for p104 (n = 173) and PIM (n = 68) analysis	Number of samples	Source of blood sample	Reference
Okavango Delta (OD) and Chobe National Park (CNP), Botswana	p104 = 64 PIM = 33	Buffalo	Jori et al. 2013
<i>OD, Ngamiland District, Botswana:</i>			Jori et al. 2013
• <i>Moremi Game Reserve (MGR)</i>	p104 = 8 PIM = 4	Buffalo	
• <i>NG30 area</i>	p104 = 23 PIM = 11		
<i>CNP, Chobe District, Botswana</i>	p104 = 33 PIM = 18	Buffalo	Jori et al. 2013
Gonarezhou National Park, Zimbabwe	p104 = 13 PIM = 5	Buffalo	Dr Calvin Gomo (pers. comm., 2010)
Kruger National Park (KNP), Mpumalanga, SA*	p104 = 43 PIM = 4	Buffalo	Sibeko, 2009
Hluhluwe-iMfolozi Park, KwaZulu-Natal, SA	p104 = 38 PIM = 6	Buffalo	Sibeko, 2009
Ladysmith, KwaZulu-Natal, SA	p104 = 6 PIM = 1	Cattle	Sibeko, 2009
Mabalingwe Game Reserve, Limpopo, SA	p104 = 6 PIM = 0	Buffalo	Sibeko, 2009
Bloemfontein, Free State, SA	p104 = 1 PIM = 1	Cattle	Sibeko, 2009
Onderstepoort Veterinary Institute (OVI), Gauteng, SA	p104 = 2 PIM = 0	Cattle	Sibeko, 2009
Itahla Game Reserve, KwaZulu-Natal, SA	p104 = 0 PIM = 10	Buffalo	Sibeko, 2009
Marakele National Park, Limpopo, SA	p104 = 0 PIM = 2	Buffalo	Sibeko, 2009
Welgevonden, Limpopo, SA	p104 = 0 PIM = 6	Buffalo	Sibeko, 2009

* SA = South Africa

4.4. Results

4.4.1. Size differentiation of the central region of the p67 *T. parva* antigen gene

The *T. parva* p67 gene could only be amplified from 13 (13.5%) of the 96 *T. parva*-positive samples (2 from CNP and 11 from OD) from Botswana, and from 9 (32.1%) of the 28 positive samples from GNP. Up to three p67 PCR product sizes (0.9, 1.0 and 1.1 kb; representative of alleles 1, 4 and 2, respectively) were obtained (Figure 4.1 and Table 4.2); the 0.8 kb allele (representative of allele 3) could not be detected. As the p67 gene is a single copy gene, multiple bands in a single sample more than likely represent the presence of several different *T. parva* genotypes. More than half of the samples (2 from CNP, 8 from OD, 3 from GNP), however, contained only a single band; suggesting the presence of a single *T. parva* genotype. The 0.9, 1.0 and 1.1 kb bands were present in, respectively, 53.8%, 23.1% and 69.2% of the Botswana buffalo samples analyzed, and 55.6%, 66.7% and 100% of the GNP samples analyzed. The most abundant allele in both Botswana and Zimbabwe was allele 2 (1.1 kb).

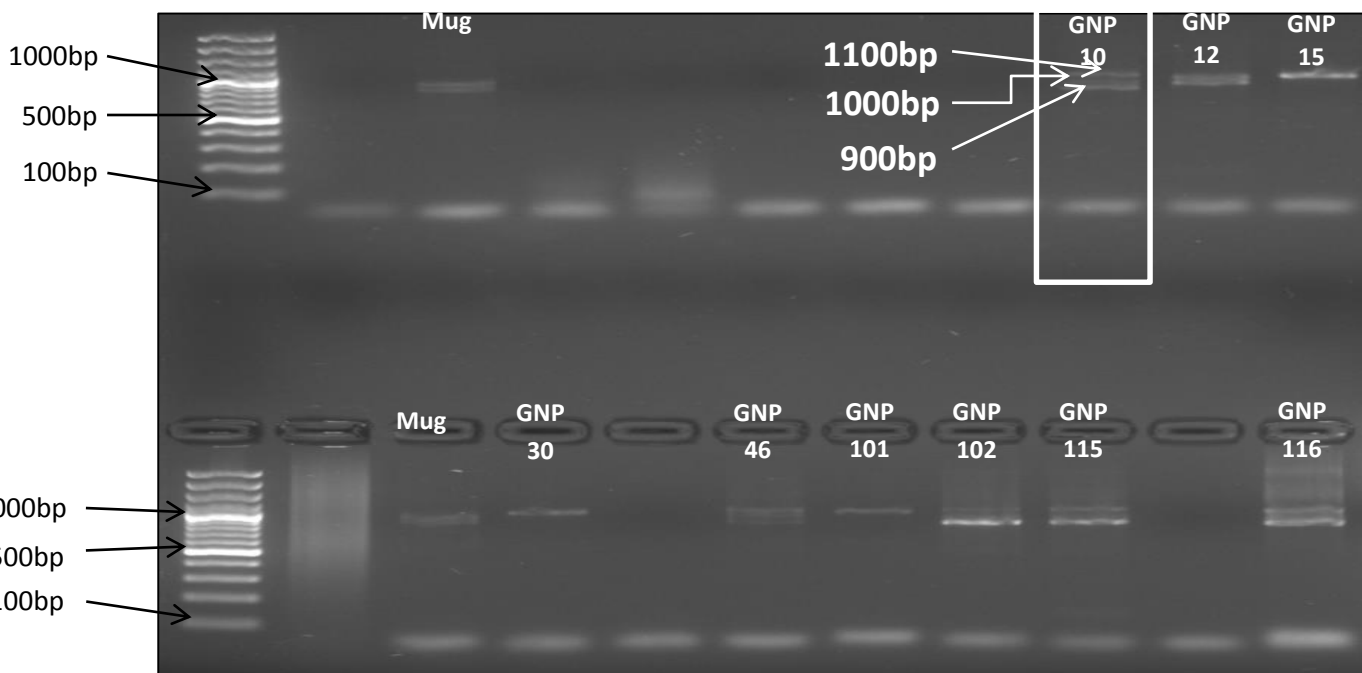


Figure 4.1: Amplicon profiles obtained from amplification of the central region of the p67 gene from *T. parva* parasites. A 100 bp molecular ladder was used and the Muguga p67 amplicon (Mug) as positive control.

Table 4.2: Results obtained from PCR amplification of the *T. parva* p67 gene.

Geographic origin	Sample designation	Band size(s) obtained from PCR amplification (Kb)
Chobe National Park (2)	CNP90	0.9
	CNP114	1.1
Okavango Delta, Botswana (11)	OD27	0.9
	OD62	0.9
	OD64	0.9
	OD65	1.1
	OD94	0.9, 1.0, 1.1
	OD100	0.9, 1.0, 1.1
	OD101	1.1
	OD102	1.1
	OD106	0.9, 1.0, 1.1
	OD108	1.1
	OD110	1.1
Gonarezhou National Park, Zimbabwe (9)	GNP10	0.9, 1.0, 1.1
	GNP12	1.0, 1.1
	GNP15	1.1
	GNP30	1.1
	GNP46	0.9, 1.0, 1.1
	GNP101	1.1
	GNP102	0.9, 1.0, 1.1
	GNP115	0.9, 1.0, 1.1
	GNP116	0.9, 1.0, 1.1

4.4.2. p104 PCR-RFLP profile analysis

A ~800 bp p104 PCR product could be amplified from 82 (85.4%) of the 96 *T. parva*-positive samples from Botswana (41 from CNP, 41 from OD), and from 13 (46.4%) of the 28 positive samples from GNP (Figure 4.2). Samples were digested using the *AluI* enzyme (Figure 4.3). From visual analysis, six samples had similar profiles to the KNP102 reference profile. The GNP86 and GNP88 profiles were visually similar to that of the *T. parva* Kiambu 5 cattle-associated profile as shown by Geysen et al. (1999). However, the Kiambu 5 profile could not be included in the subsequent cluster analysis as control DNA was not available for PCR-RFLP analysis.

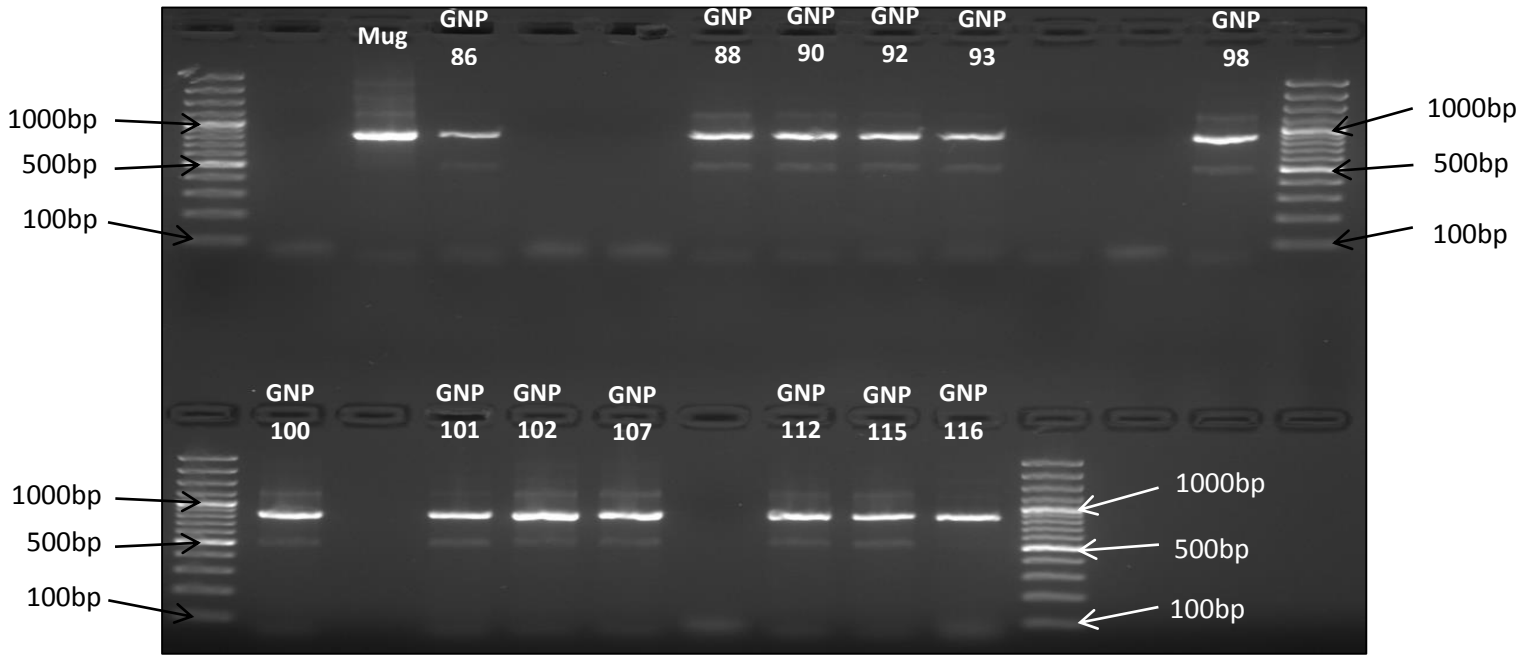


Figure 4.2: Gel image illustrating amplicons obtained from amplification of the variable region of the p104 gene from *T. parva* parasites. Sample numbers are indicated above each lane; a 100 bp molecular ladder was included as well as the Muguga positive control p104 amplicon (Mug).

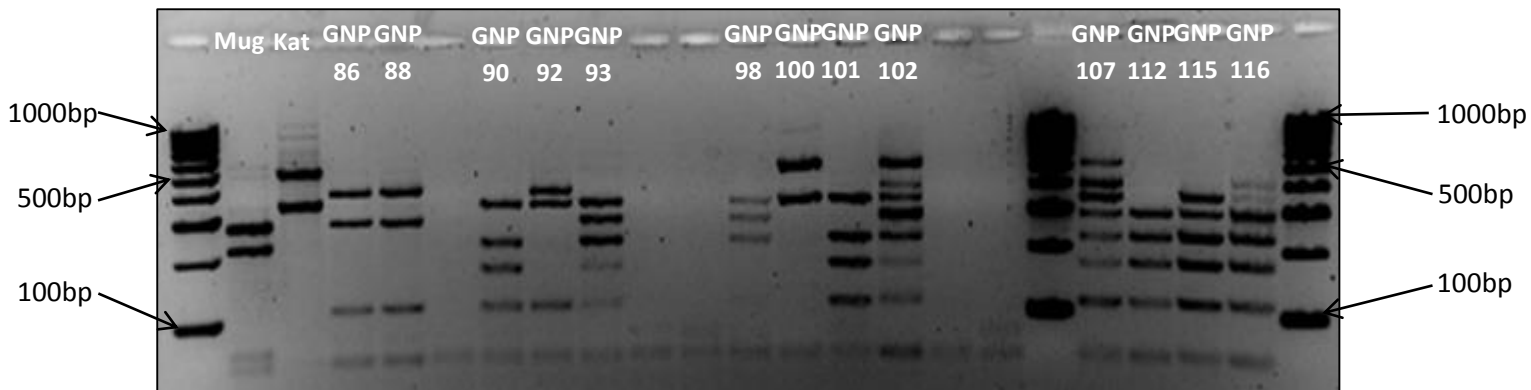
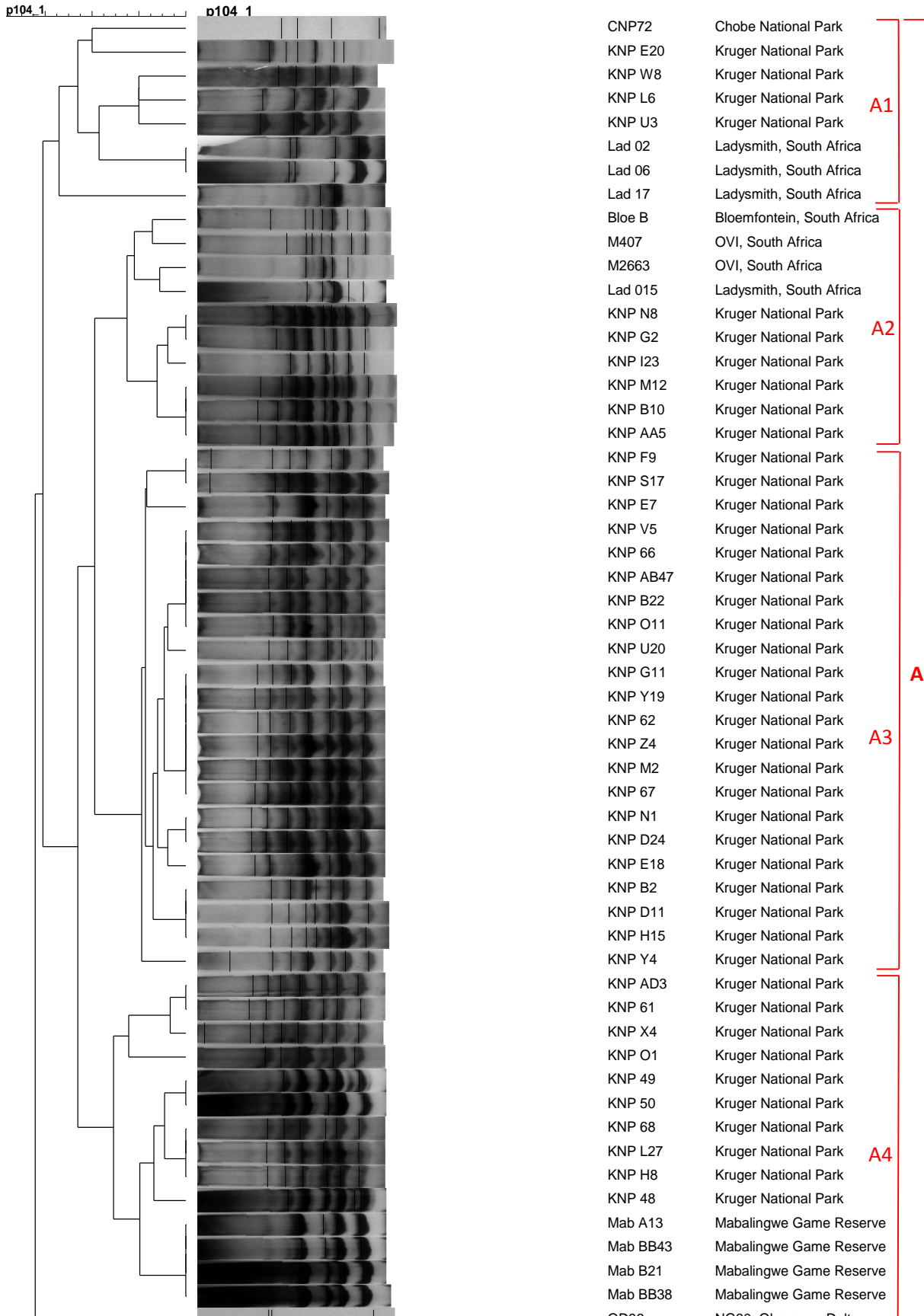
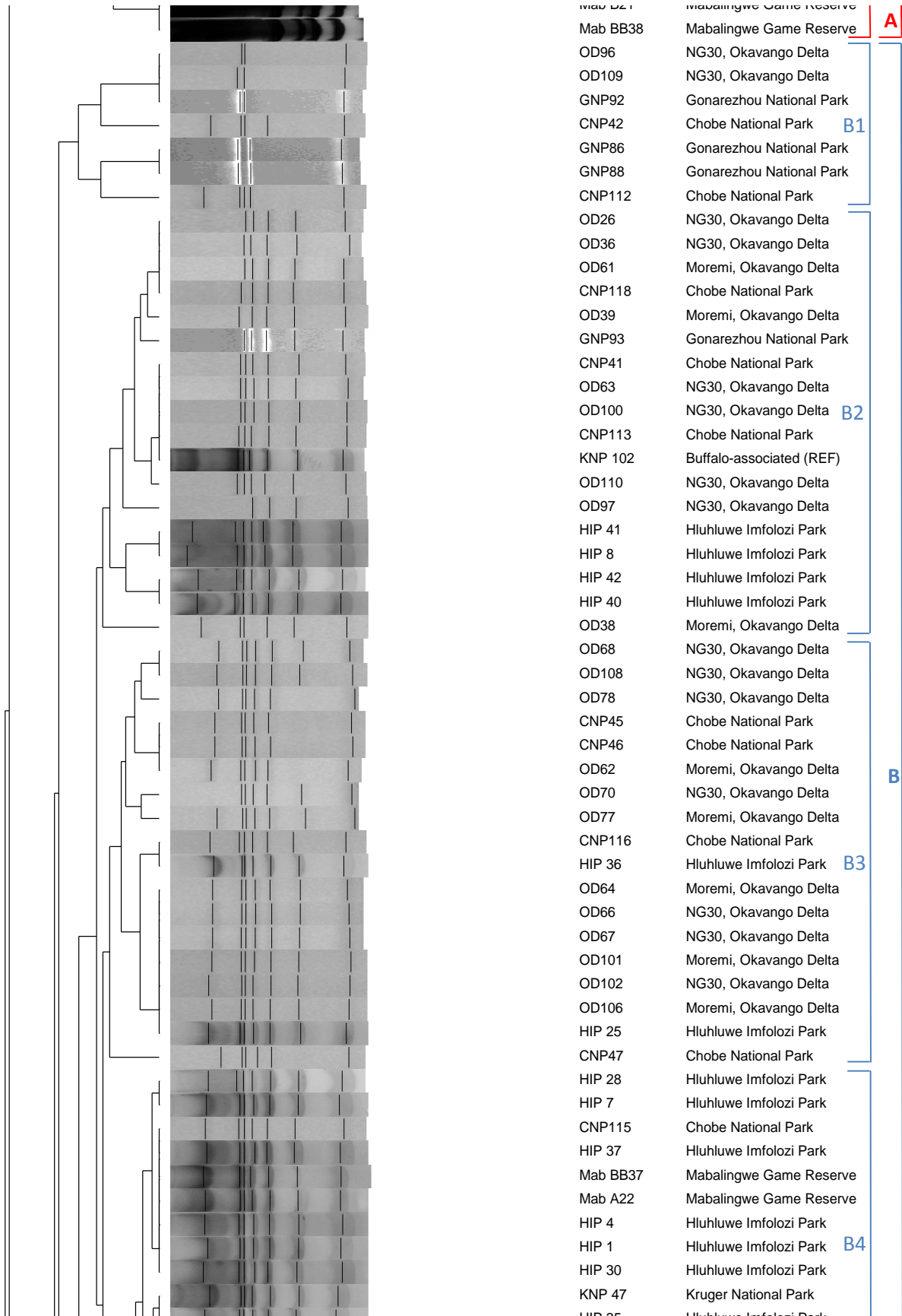


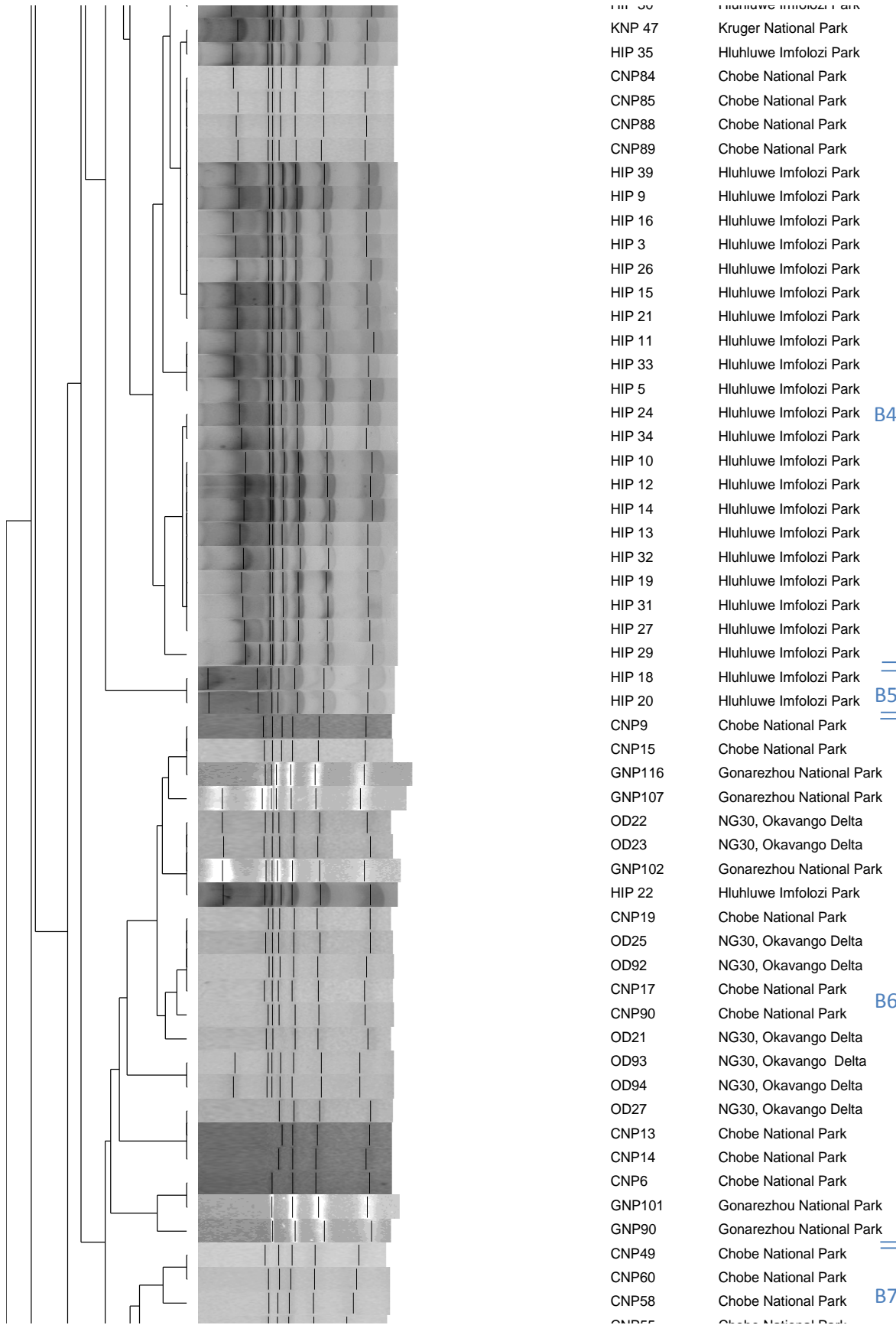
Figure 4.3: Gel image illustrating the representative *AluI* digested p104 PCR-RFLP profiles obtained. Sample numbers are indicated above each lane; 100 bp molecular ladders as well as the Muguga (Mug) and Katete (Kat) reference profiles were included.

A UPGMA dendrogram was subsequently constructed using the p104 PCR-RFLP profiles obtained. Buffalo and cattle *T. parva* p104 PCR-RFLP profiles, previously generated by Sibeko et al. (2009), were also included in the analysis (Table 4.1). In a number of samples (20), a PCR-RFLP profile could not be obtained and these were excluded from further analysis. Cluster analysis of the p104 PCR-RFLP profiles using the Bionumerics software, identified three cluster groups, A, B and C; clustering mostly according to geographic origin of the samples (Figure 4.4). Cluster A consisted of four sub-groups. Only one buffalo sample from the CNP (1/33) was identified in Cluster A. The

remainder of the samples comprised of South African buffalo and cattle samples; 42 buffalo from the Kruger National Park (KNP) (42/43), four buffalo from Mabalingwe Game Reserve (4/6), six cattle from Ladysmith (4/6) and another three cattle samples, one from Bloemfontein (1/1) and two from the Onderstepoort Veterinary Institute (2/2). Cluster B consisted of eight sub-groups. Twelve of 13 GNP (12/13) buffalo, all OD (31/31) buffalo profiles and mostly all CNP (29/33) buffalo profiles clustered in Cluster B. The remainder consisted of buffalo from South Africa; all profiles from Hluhluwe-iMfolozi (38/38), one from KNP (1/44) and two from Mabalingwe Game Reserve (2/6). Cluster C contained two samples from Ladysmith (2/6) and three from CNP (3/33) even though CNP profiles were not that similar to those from Ladysmith visually. Profiles obtained from the CNP, GNP and OD grouped together in sub-groups 1, 2 and 6. CNP profiles further were identified in 3, 4, 7 and 8, GNP in 7 and 8 while OD was only identified in sub-group 3. One profile from GNP (GNP100) and the Katete positive control profile did not group in any of the clusters but had identical profiles (Figure 4.4).







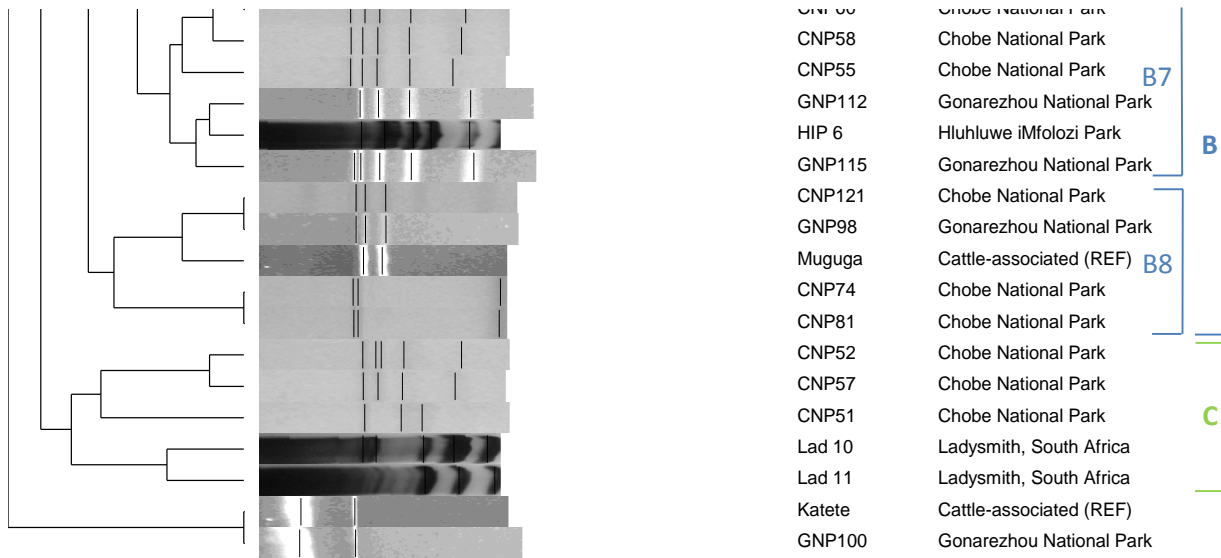


Figure 4.4: UPJMA dendrogram for the *T. parva* p104 PCR-RFLP profiles based on the Dice coefficient analysis. Known cattle-associated *T. parva* parasite (Muguga and Katete) and buffalo-associated *T. parva* (KNP102) were included in the analysis as reference profiles.

4.4.3. PIM PCR-RFLP profile analysis

PIM gene amplicons could be obtained from 56 (58.3%) of the Botswana samples (32 from CNP and 24 from OD) and 5 (17.9%) of the Zimbabwe samples, respectively. PCR products from Botswana and Zimbabwe delivered a variety of band sizes (Figure 4.5 and Table 4.3). The PIM gene is a single copy gene so multiple bands indicated samples with more than one infection of *T. parva*. Samples were digested using *Bcl1* enzyme (Figure 4.6). The profile of CNP57 visually resembled that of the Zam 5 profile isolated previously in Zambia (Geysen et al. 1999). Visually, samples OD97 and OD100 had a similar profile to that of the Kiambu5-cattle associated PIM profile from Kenya (Geysen et al. 1999). The Kiambu 5 and Zam 5 profiles could not be included in the subsequent cluster analysis due to the unavailability of control DNA to generate PCR-RFLP profiles.

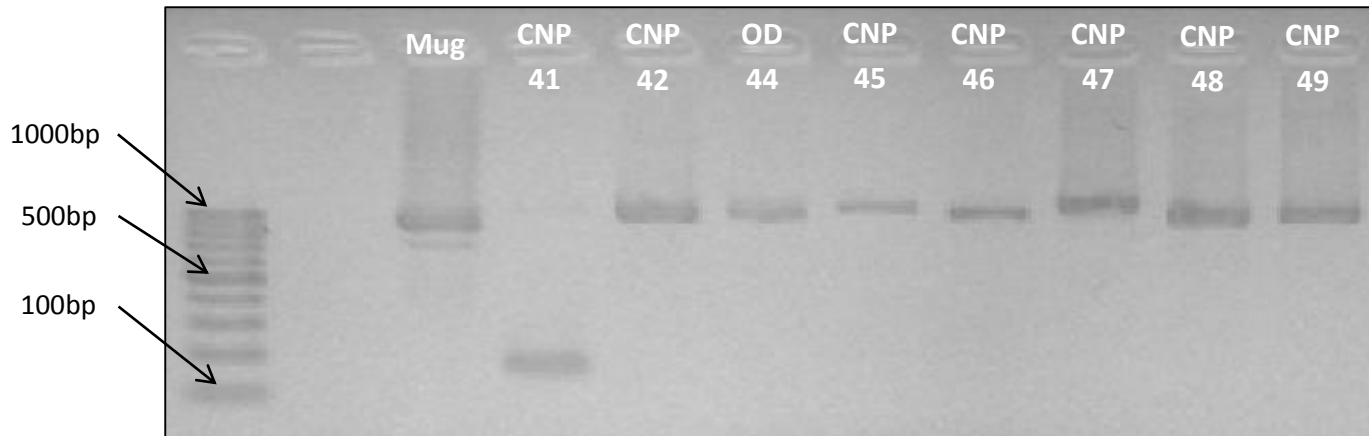


Figure 4.5: Gel image illustrating amplicons obtained from amplification of the variable region of the PIM gene from *T. parva* parasites. Sample numbers are indicated above each lane; a 100bp molecular ladder was included as well as the Muguga (Mug) positive control PIM amplicon.

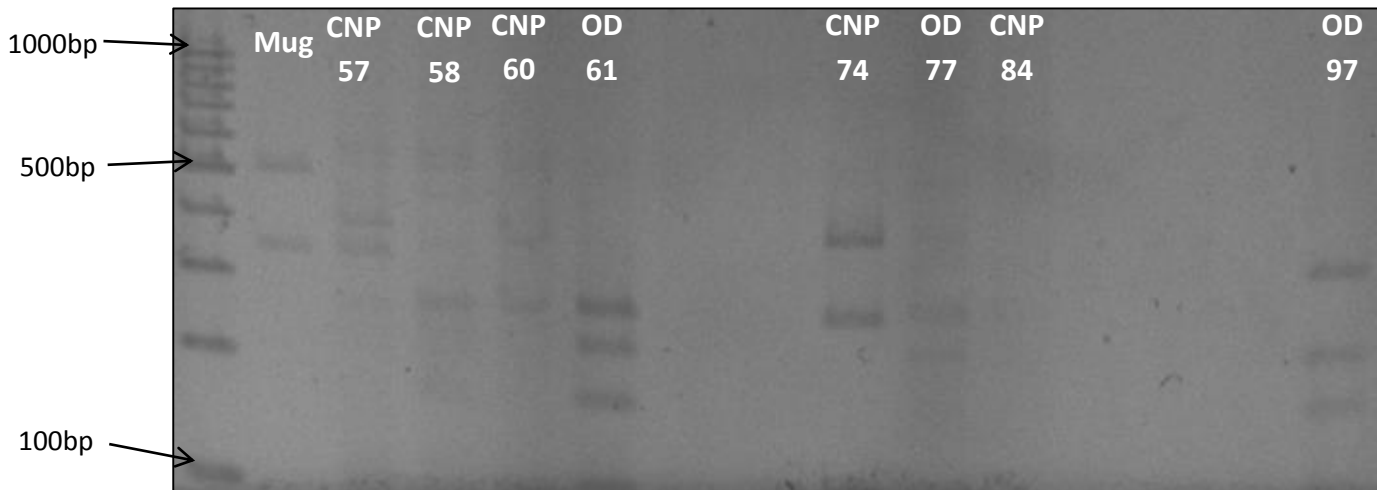


Figure 4.6: Gel image illustrating the representative *BclI* digested PIM PCR-RFLP profiles obtained. Sample numbers are indicated above each lane; a 100 bp molecular ladder as well as the Muguga (Mug) reference profile was included.

Table 4.3: Amplicon sizes obtained from PCR amplification of the *T. parva* PIM gene.

Geographic origin	Sample designation	Band size(s) obtained from PCR amplification (Kb)
Chobe National Park (32)	CNP7	0.1
	CNP5, CNP16, CNP41	0.15
	CNP15, CNP90*	0.2
	CNP115	0.25
	CNP3*, CNP121*	0.5
	CNP58*, CNP84*	0.8
	CNP11*, CNP48, CNP60, CNP74, CNP88,	0.9

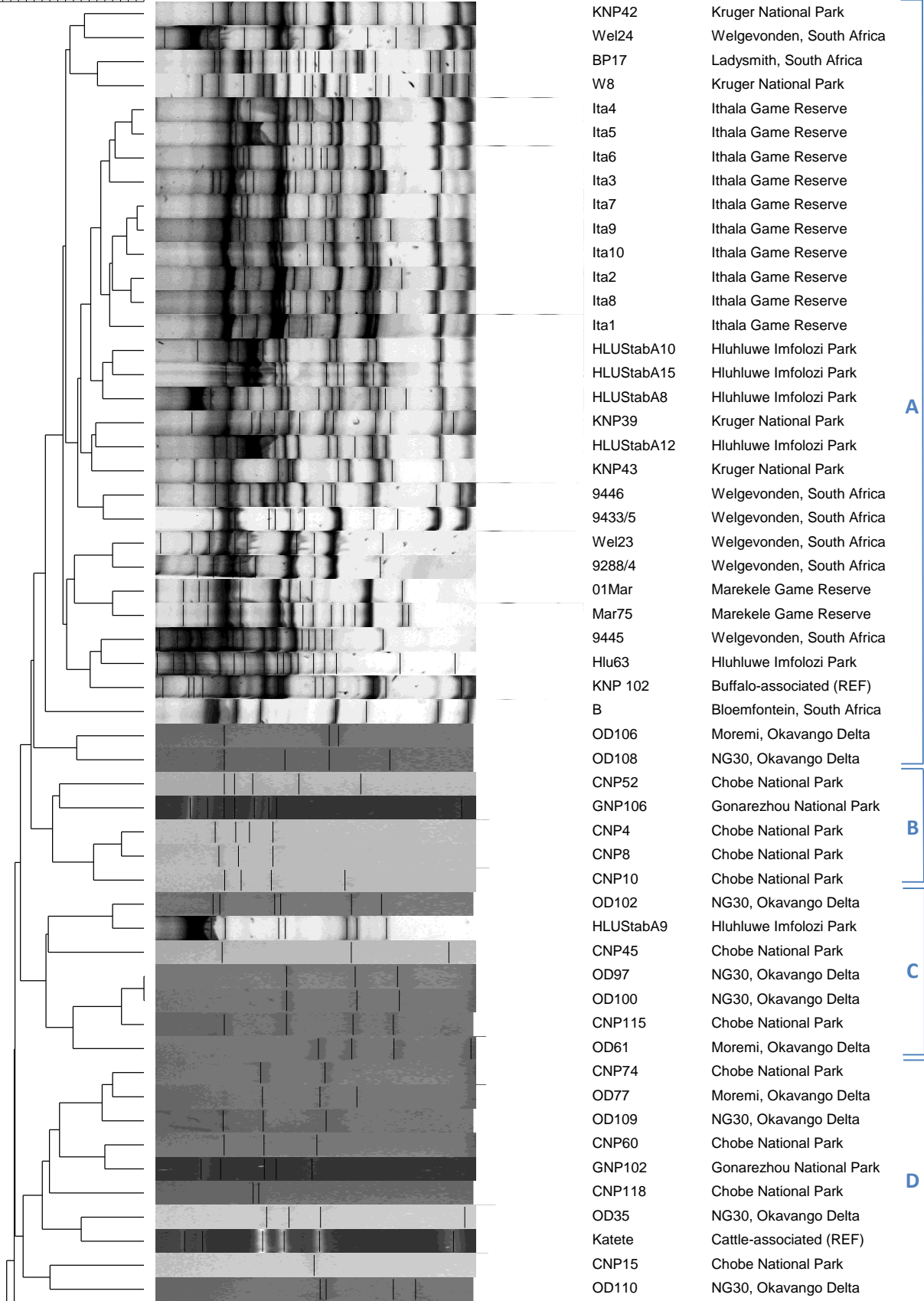
	CNP90*	
	CNP10, CNP11*, CNP42, CNP46, CNP49, CNP51, CNP52, CNP55, CNP57, CNP58*, CNP84*, CNP118, CNP121*	1.0
	CNP1, CNP2, CNP3*, CNP4, CNP8, CNP17, CNP45, CNP47	1.1
Okavango Delta (24)	OD100*	0.25
	OD97*	0.6
	OD32, OD35, OD61, OD62, OD100*, OD101, OD102, OD106*	0.9
	OD22, OD24, OD29, OD34, OD36, OD38*, OD39, OD44, OD63, OD77, OD93*, OD106*, OD108, OD109, OD110	1.0
	OD37, OD97*	1.1
	OD38*, OD93*	1.2
Gonarezhou National Park, Zimbabwe (5)	GNP106*	0.8
	GNP106*	1.1
	GNP96, GNP102, GNP103, GNP115	1.2

*Samples represented by more than one band

A UPGMA dendrogram was constructed using the PIM PCR-RFLP profiles obtained. Buffalo and cattle *T. parva* PIM PCR-RFLP profiles, previously generated by Sibeko et al. (2009), as well as the cattle-derived Katete and Muguga *T. parva* reference profiles were included in the analysis (Table 4.1). In a number of samples (24), a PCR-RFLP profile could not be obtained and these were excluded from further analysis. Cluster analysis identified six clusters, A, B, C, D, E and F. Cluster A contained mostly South African buffalo (n = 29), two cattle samples (Bloemfontein and Ladysmith) and two samples from OD. The remainder of OD, CNP and GNP were distributed throughout clades B to F. Two samples (CNP5 and CNP16) probably were not digested completely and only gave a single band, these grouped in clade F. An exception was one sample from Hluhluwe that grouped in clade C with samples from Botswana (Figure 4.7). The Muguga PCR-RFLP profile clustered in group E together with six samples from CNP, five from OD and three from GNP even though the Muguga profile did not have a very similar profile to any of these samples. The cattle-derived Katete *T. parva* stock from Zambia (Geysen, 2000) clustered in group D with eight samples from Botswana and one from GNP.

PIM clones_150909

PIM clones_150909



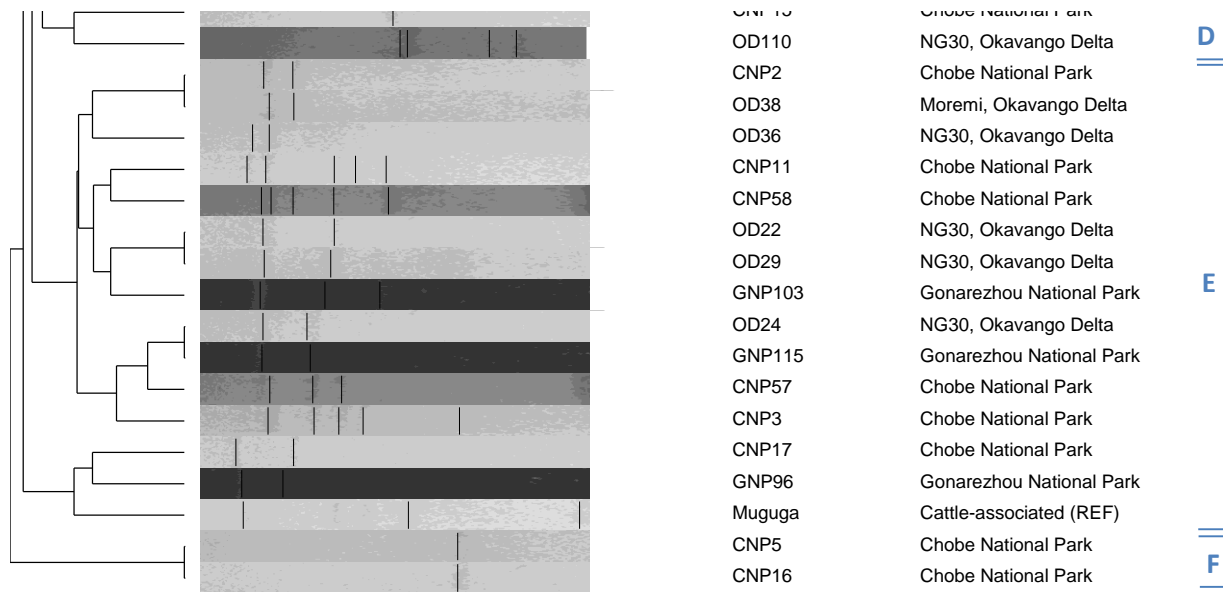


Figure 4.7: UPJMA dendrogram for the *T. parva* PIM PCR-RFLP profiles based on the Dice coefficient analysis. Known cattle-associated *T. parva* parasite (Muguga and Katete) and buffalo-associated *T. parva*, KNP102 were included in the analysis as reference profiles.

4.5. Discussion

Analysis of p67 PCR amplicon profiles indicated that three p67 alleles (alleles 1, 2 and 4) are present in *T. parva* parasites circulating in buffalo from two distinct northern Botswana buffalo populations and one population from southern Zimbabwe. The most dominant allele found was the buffalo-derived allele 2 found in 69.2% and 100% of the buffalo from Botswana and Zimbabwe, respectively. The cattle-derived allele 1 was detected in 53.8% and 55.6% of the buffalo from Botswana and Zimbabwe, respectively. This corresponded with the findings of Sibeko et al. (2009) where allele 2 was obtained from 82%, and allele 1 from 77% of the South African buffalo investigated. In a study by Elisa et al. (2014), all four p67 alleles were found in buffalo and cattle in the Serengeti and Ngorongoro areas, northern Tanzania. Alleles 1 and 2 were found as mixed profiles in 89% and 88% of buffalo from Serengeti and Ngorongoro, respectively.

The p67 allele 3 could not be detected in any of the buffalo investigated in our study. Similarly, Elisa et al (2014) could not detect allele 3 in any of the buffalo samples from northern Tanzania. However, in the Sibeko et al. (2009) study, allele 3 was detected in 61% of South African buffalo. The authors speculated that *T. parva* parasites carrying the p67 allele 3 could be transmitted to cattle since allele 3 was identified in a naturally infected bovine diagnosed with ECF in the southern province of Zambia (Geysen, 2000).

Allele 4 was detected in 23.1% and 66.7% of the buffalo from Botswana and Zimbabwe, respectively. Sibeko et al. (2009) found allele 4 in 39% of the South African buffalo investigated. In the study by Elisa et al. (2014), none of the buffalo samples had allele 4; however, all of the Serengeti cattle samples had allele 4, which was also observed in 57% of the Ngorongoro cattle samples. The authors speculated that this allele possibly originated in the Serengeti or was introduced by bringing to the area infected cattle from Ngorongoro. Extensive diversity of cattle-derived *T. parva* has been observed within the Ngorongoro area; since there is no limitation of cattle movement between Serengeti and Ngorongoro, this may increase the genotype flow in these two areas. In addition, it will be interesting to establish whether parasites with alleles 3 and 4 are responsible for ECF cases in East Africa. This could also have severe implications in Botswana and Zimbabwe should buffalo carrying *T. parva* with these alleles come into contact with naïve cattle.

The phylogenetic analysis of the p67 gene sequences obtained by Sibeko et al. (2009) indicated that parasites containing p67 alleles 3 and 4 seem to have evolved separately from the cattle- and buffalo-derived parasites carrying alleles 1 and 2. This is interesting given the extensive recombination known to occur between *T. parva* parasites in the tick vector (Nene et al., 1998). The authors further speculated that the cattle-derived p67 *T. parva* alleles evolved from buffalo-derived p67 alleles. This supports the belief that *T. parva* is originally a buffalo parasite (Uilenberg, 1981; Young, 1981; Norval et al., 1992) and the hypothesis that selection of a subpopulation of *T. parva* parasites resulted in ECF (Young, 1981; Conrad et al., 1989).

Cluster analysis of the *T. parva* p104 PCR-RFLP profiles identified two groups correlating largely with the geographic origin of the samples and a third that grouped apart from the first two which were more similar to each other; this confirmed the groupings previously found by Sibeko et al. (2011). Profiles obtained from buffalo from Botswana and Zimbabwe clustered mostly in Cluster B together with the homogeneous profiles obtained from buffalo from Hluhluwe-iMfolozi game park, South Africa (Sibeko et al., 2011). This would suggest that, as in the case with Hluhluwe-iMfolozi, a less diverse population of *T. parva* parasites are circulating in the CNP, OD and GNP. Only one profile obtained from CNP buffalo clustered with the more heterogeneous profiles, and thus a more diverse population of *T. parva* parasites previously identified in buffalo from KNP, South Africa (Cluster A) (Sibeko et al., 2011).

Two buffalo samples, CNP121 and GNP98, had a cattle-type *T. parva* p104 RFLP profile almost identical to that of *T. parva* Muguga; sequence data is, however, required to confirm this finding. In contrast, Sibeko et al. (2011) could not identify Muguga-like cattle-type p104 PCR-RFLP profiles in any of the South African buffalo samples investigated. Another sample, GNP100, had an identical profile to the *T. parva* Katete profile but these two grouped completely apart from the rest of the

dataset. Three CNP buffalo samples (CNP51, CNP52 and CNP 57) grouped with Lad10 and Lad11 buffalo-type p104 profiles obtained from cattle from a farm in Ladysmith, South Africa (Sibeko et al. 2011). The authors (Sibeko et al., 2011) speculated that *T. parva* parasites circulating in cattle on the Ladysmith farm may have been transmitted from buffalo to cattle, although there was no evidence of any contact between these cattle and buffalo (Thompson et al, 2008). Furthermore, five buffalo samples (CNP41, CNP113, OD63, OD100 and OD110) were very similar to the KNP102 p104 PCR-RFLP profile originally obtained from buffalo KNP102 (Sibeko et al. 2008). This buffalo, which had a multiple infection of *T. parva* parasites possessing all four p67 alleles, was previously used in a tick transmission experiment where the infected bovine eventually died of Corridor disease (data not published).

Sibeko et al. (2011) further showed that no significant correlation could be found between cluster analysis of p104 PCR-RFLP profiles and the phylogeny obtained from p104 gene sequence analysis. Due to mixed infections, PCR-RFLP cluster analysis represents an overall profile of the *T. parva* strains present in a sample, whereas p104 gene sequence analysis represents individual *T. parva* strains. Also, in a mixed infection one would expect that PCR-RFLP cluster groups would be overwhelmed by profiles of the most dominant parasites. Ideally PCR-RFLP cluster analysis profiles should be produced from clones; and one would expect that it will correlate better with p104 gene sequence analysis.

An attempt was made to use p104 sequencing data from Sibeko et al. (2011) to determine if samples from this study were more likely to be buffalo-type or cattle-type isolates through comparison of sequencing and clustering data. Like Sibeko et al. (2011), the correlation between sequence data and PCR-RFLP's wasn't good and we were unable to identify the most prominent isolate type. This is probably the effect of mixed genotypes in the PCR-RFLP analysis versus single clones in the sequencing data.

The *T. parva* PIM gene is very polymorphic and previous studies have shown that PIM PCR-RFLP profiles obtained from buffalo are more complex than those obtained from cattle (Geysen, 2000; Sibeko et al. 2011). Examination of the profiles from this study revealed a high level of heterogeneity with six different clades formed in the cluster analysis. This is in contrast with the p104 PCR-RFLP profile data that indicated that a less diverse populations of *T. parva* parasites are circulating in the CNP, OD and GNP. Sibeko et al. (2009) reported a relative homogeneity between samples originating from Hluhluwe-iMfolozi, Mabalingwe and Ithala game parks. The authors speculated that small populations of buffalo may have a limited genetic diversity in *T. parva*.

In a study by Elisa et al. (2014), they were able to identify six distinct PIM profiles for buffalo-derived isolates and five for cattle-derived isolates in their analysis. This was not the case in this study where no distinct profiles could be identified; in addition our study did not contain any cattle-derived samples. This may be because of the high genetic diversity of *T. parva* in these buffalo populations or the fact that samples contain more than one parasite whereby other underrepresented PIM profiles present in the sample were masked. Another shortfall might be that our sample size was too small to identify distinct profiles.

In addition, an attempt to use PIM sequencing data from Sibeko et al. (2011) to identify whether samples from this study were more likely to be buffalo-type or cattle-type isolates through comparison of sequencing and clustering data was not successful.

Differentiation between buffalo-derived and cattle-derived isolates using the p67, p104 and/or PIM genes were unsuccessful, but it still gave an indication of the diversity of *T. parva* parasites that circulate in buffalo from Botswana and Zimbabwe. An important factor that needs to be taken into account when looking at *T. parva* genetic diversity is the high level of buffalo movement in southern Africa, whether it be natural migration or artificial translocations (Smitz et al., 2014). It is expected that isolated buffalo populations should share similar parasite genetic patterns but translocations of buffalo may have introduced new *T. parva* diversity into that population. Translocations such as these have been identified in Zimbabwe (Smitz et al., 2014). This could have led the buffalo populations in this study to have a genetically more diverse *T. parva* circulating in them.

4.6. Conclusion

We were not successful in differentiating between buffalo-derived and cattle-derived isolates using the p67, p104 and/or PIM genes; however, important information was gathered regarding the diversity of *T. parva* parasites that circulate in buffalo from Botswana and Zimbabwe. To improve on this study, a larger sample set would be required that includes cattle samples from the different areas as well as gene sequence data of single isolates.

4.7. References

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Chapter 5

Concluding remarks

This study illustrates the diversity of haemoparasites present in African buffalo from northern Botswana and Zimbabwe and highlights the role of African buffalo as a sentinel species for tick-borne pathogens of livestock. Our results indicate the significance of the African buffalo as reservoir host for important tick-borne haemoparasites that can cause severe disease in cattle. Important tick-borne haemoparasites identified in this study included: *T. parva*, *A. marginale*, *B. bovis* and *E. ruminantium*. Results also suggest that qPCR and IFAT are more efficient in detecting buffalo exposed to *T. parva* than the RLB test. Although we were not successful in differentiating between buffalo-derived and cattle-derived *T. parva* isolates using the p67, p104 and/or PIM gene data, important information was gathered on the diversity of *T. parva* parasites that circulate in buffalo from Botswana and Zimbabwe. This study should contribute to raise awareness among veterinary authorities regarding the potential occurrence of these parasites in cattle so that appropriate control and surveillance protocols, taking into account the presence of infected wildlife reservoirs in those areas, can be designed at the wildlife-livestock interface.

APPENDIX I

RESEARCH

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Tick-borne haemoparasites in African buffalo (*Syncerus caffer*) from two wildlife areas in Northern Botswana

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Abstract

Background: The African buffalo (*Syncerus caffer*) is a host for many pathogens known to cause economically important diseases and is often considered an important reservoir for livestock diseases. Theileriosis, heartwater, babesiosis and anaplasmosis are considered the most important tick-borne diseases of livestock in sub-Saharan Africa, resulting in extensive economic losses to livestock farmers in endemic areas. Information on the distribution of tick-borne diseases and ticks is scarce in Northern Botswana. Nevertheless, this data is necessary for targeting surveillance and control measures in livestock production at national level.

Methods: In order to address this gap, we analyzed 120 blood samples from buffalo herds for the presence of common tick-borne haemoparasites causing disease in livestock, collected in two of the main wildlife areas of Northern Botswana: the Chobe National Park (CNP, n = 64) and the Okavango Delta (OD, n = 56).

Results: Analysis of the reverse line blot (RLB) hybridization assay results revealed the presence of *Theileria*, *Babesia*, *Anaplasma* and *Ehrlichia* species, either as single or mixed infections. Among the *Theileria* spp. present, *T. parva* (60%) and *T. mutans* (37%) were the most prevalent. Other species of interest were *Anaplasma marginale* subsp. *centrale* (30%), *A. marginale* (20%), *Babesia occultans* (23%) and *Ehrlichia ruminantium* (6%). The indirect fluorescent antibody test (IFAT) indicated 74% of samples to be positive for the presence of *T. parva* antibodies. Quantitative real-time PCR (qPCR) detected the highest level of animals infected with *T. parva* (81% of the samples). The level of agreement between the tests for detection of *T. parva* positive animals was higher between qPCR and IFAT (kappa = 0.56), than between qPCR and RLB (kappa = 0.26) or the latter and IFAT (kappa = 0.15).

Conclusions: This is the first report of tick-borne haemoparasites in African buffalo from northern Botswana, where animals from the CNP showed higher levels of infection than those from OD. Considering the absence of fences separating wildlife and livestock in the CNP and the higher levels of some parasite species in buffalo from that area, surveillance of tick-borne diseases in livestock at the interface in the CNP should be prioritized.

Keywords: Botswana, African buffalo, Haemoparasites, Tick-borne diseases, *Theileria*, *Babesia*, *Anaplasma*, *Ehrlichia*, Reverse line blot hybridization assay, Real-time PCR, IFAT

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Background

Theileriosis, babesiosis, anaplasmosis and heartwater are considered to be the most important tick-borne diseases (TBDs) of livestock in sub-Saharan Africa, resulting in extensive economic losses to farmers in endemic areas. The African buffalo (*Syncerus caffer*) is the natural reservoir host of *Theileria parva*, which is transmitted by the tick species, *Rhipicephalus appendiculatus*, *R. zambeziensis* and *R. duttoni* [1,2]. *T. parva* causes East Coast fever (ECF), which occurs in eastern and central Africa. ECF was introduced into southern Africa in the early 1900s through cattle importation from East Africa and was eradicated from South Africa in the 1950s [3,4]. *T. parva* also causes Corridor Disease, which is still prevalent in South Africa in areas where buffalo and cattle share grazing grounds in the presence of its tick vectors. It is a controlled disease in South Africa because of a concern that ECF might recur [5,6]. Although *T. parva* distributions have been described in Mozambique [7], Zambia [8] and Zimbabwe [3], no information on the distribution of *T. parva* is available for many other southern African countries, including Botswana.

In addition to *T. parva*, buffalo are also thought to be the original reservoir host of other non-pathogenic, mildly pathogenic and benign *Theileria* species namely, *Theileria mutans*, *Theileria velifera*, *Theileria buffeli*, *Theileria* sp. (buffalo) [9,10] and *Theileria* sp. (bougasvlei) [11,12]. *Theileria* parasites usually occur as mixed infections in buffalo and cattle. Although the benign and non-pathogenic forms do not have any significant economic importance, their presence could interfere with the interpretation of results obtained in some diagnostic tests designed to diagnose the pathogenic *T. parva*. Diagnostic tests for *T. parva* include microscopic examination of blood smears for the presence of piroplasm and schizonts, and serological methods such as the indirect fluorescent antibody test (IFAT) which is routinely used in South Africa for *T. parva* antibody detection in “disease free” buffalo [13,14]. Molecular diagnostic methods detect specific parasite sequences in DNA extracts from blood or tissue samples. The reverse line blot (RLB) hybridization assay makes use of polymerase chain reaction (PCR) amplification of haemoparasite small subunit ribosomal RNA genes (srRNA) which are screened with group- and species-specific probes for the simultaneous detection and identification of haemoparasites in mixed infections [15]. To date, the most sensitive molecular test for the detection of *T. parva* is a quantitative real-time PCR (qPCR) using hybridization probe chemistry, where the central region of the parasite 18S rRNA gene is amplified and the presence of *T. parva* is confirmed by melting curve analysis [16]. It is currently routinely used to test for *T. parva* infections in buffalo and cattle in South Africa as part of the Corridor disease control strategy.

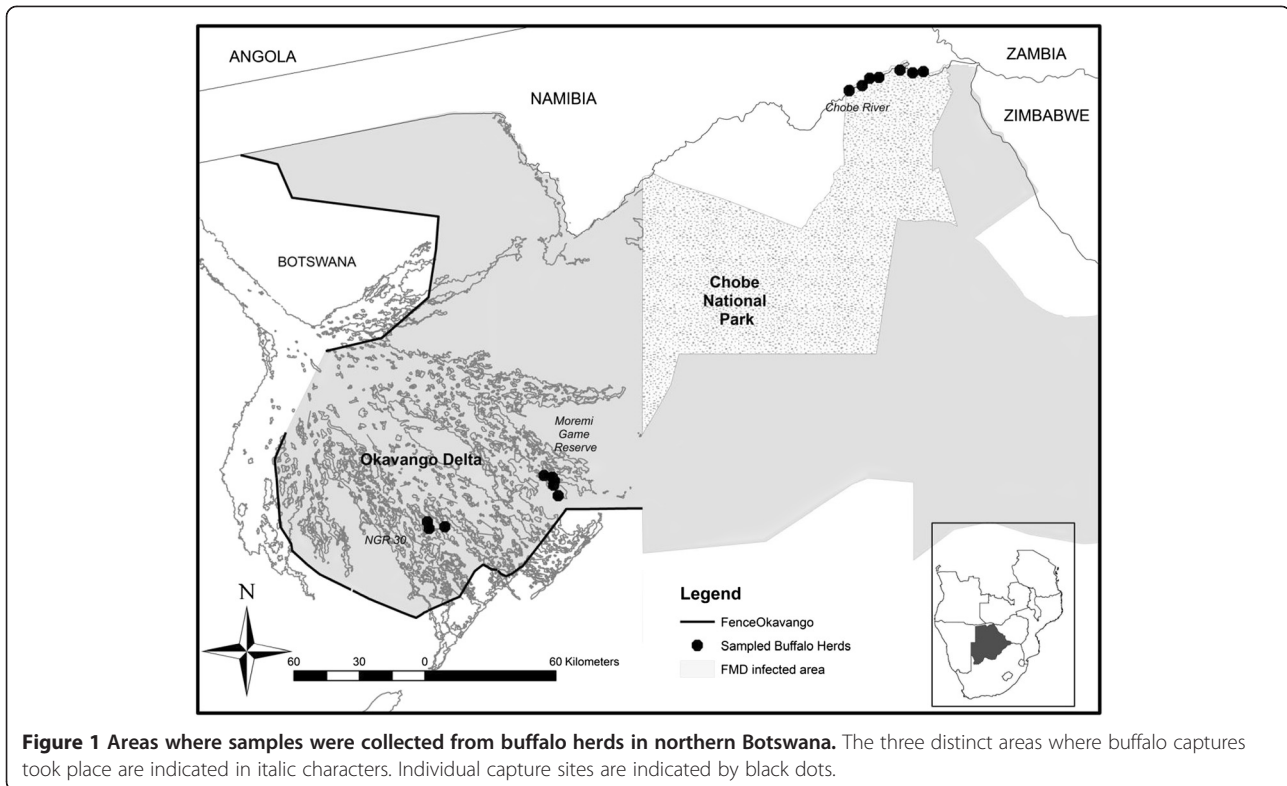
It has been shown that African buffalo are also carriers of a number of other tick-borne parasites which are detrimental to livestock including *Ehrlichia ruminantium*, *Babesia bigemina*, *B. bovis*, *Anaplasma marginale* and *A. marginale* subsp. *centrale* [17-19]. Although buffalo show no disease symptoms, as reservoir hosts, they may represent a threat to the livestock industry. *Ehrlichia ruminantium*, an intracellular rickettsial bacterium, is the causative agent of heartwater (cowdriosis) and is transmitted by three-host ticks belonging to the genus, *Amblyomma* [20]. *Babesia bigemina* and *B. bovis* cause bovine babesiosis, commonly known as redwater fever. Tick vectors for these parasites include *Rhipicephalus microplus* (formerly *Boophilus microplus*) and *Rhipicephalus annulatus* (formerly *Boophilus annulatus*). It is believed that *Babesia* is the second most common blood parasite after trypanosomes representing a significant health risk for cattle [21]. *Anaplasma marginale* causes bovine anaplasmosis which is characterized by the infiltration of the host's red blood cells. It can be transmitted to other hosts through mechanical transmission but the most important mode of transmission is via tick bites, the main tick vector being *R. decoloratus* (formerly *Boophilus decoloratus*) [22,23]. *Anaplasma marginale* subsp. *centrale* causes a milder form of anaplasmosis, and is used in a live blood vaccine in many countries, including South Africa [24].

Generally speaking, publications on significant tick-borne haemoparasites in Botswana are limited [25-28] and there are no published reports of the presence of *T. parva* in livestock in this country. In addition, published literature on the occurrence of pathogens in buffalo populations from Botswana is very scarce [29]. Therefore, the goal of this study was to determine the prevalence of tick-borne parasites circulating in two distinct buffalo populations from Northern Botswana using different diagnostic methods and to use these data to compare the performance of those tests in detecting *T. parva* in buffalo.

Methods

Buffalo sampling

The Chobe National Park (CNP) and Okavango Delta (OD) are located in two different districts of Northern Botswana (Chobe and Ngamiland Districts, respectively) and represent the largest wildlife areas in this part of the country. They are both integrated in the Foot and Mouth Disease infected area, a large part of the northern region of Botswana devoted to wildlife conservation in which buffalo populations are separated from the primary cattle export and buffer zones by the use of veterinary cordon fences (Figure 1). The Chobe, Zambezi and Okavango rivers are the largest in the region, providing abundant water throughout the year. Rainfall is strongly



seasonal, occurring mostly from December to April (wet season). Vegetation consists mainly of deciduous dry woodland and scattered grasslands. Wildlife abundance is fundamentally dependent on rainfall and water availability and varies cyclically throughout the years [30]. The CNP encompasses 10 700 km² of savannah grassland. The boundaries of CNP are natural, the Chobe river in the north constituting the natural border between Botswana and Namibia. There is no physical barrier preventing contacts between cattle and wildlife and the main water source for the animals in that area is the Chobe river which is exposed to seasonal variations of water levels. The OD encompasses 16 000 km² and contrary to the CNP, it is delineated from livestock areas by a double veterinary cordon fence to prevent contacts between wildlife and cattle [29], and it is largely flooded throughout the year. According to the last available wildlife census from Northern Botswana, buffalo populations and densities are estimated at 31 500 individuals and 0.94 individuals/km² in OD and 7 500 individuals and 0.23 individuals/km² in the CNP [31].

The sampling process was opportunistic and details of the capture approach were described [29]. During the capture process, blood samples were collected from a total of 120 individual buffalo. In the CNP, buffalo were captured along the Chobe river and blood samples were collected from 64 individual buffalo belonging to seven distinct herds. In the OD, 8 buffalo herds were sampled

in two different management units: the Moremi Game Reserve (MGR) (n = 18 individuals, 4 herds) and in the NG30 area (n = 38 individuals, 4 herds) (Figure 1). Buffalo densities in those locations were estimated at 1.88 buffalo/km² for the Chobe river, 1.37 buffalo/km² for the MGR and 3.55 buffalo/km² for the NG30 area of the OD [31]. The sex and age of the animals were recorded. Age was measured according to dentition; animals younger than 3 years were considered as young and animals older than 3 years were considered to be adults. Whole blood samples were obtained from the jugular vein, maintained in refrigeration and sent to the Botswana National Veterinary Laboratory in Gaborone. There, they were centrifuged at 1500 xg for 15 minutes. Sera was then harvested with a pipette and stored frozen at -20°C, until the samples were ready to be sent to the Agricultural Research Council–Onderstepoort Veterinary Institute (ARC-OVI) in South Africa for analysis.

Blood smears and DNA extraction

A total of 120 thin layer smears were prepared in the field from a blood drop of the ear subliminal vein of the captured buffalo (two individuals were missed). Those were dried in the sun and fixed with methanol. At the laboratory, each smear was stained with Giemsa dye following the standard procedures. Genomic DNA was extracted from the Giemsa-stained slides using the QIAamp DNA mini kit (Qiagen) following the manufacturer's

protocol. The DNA was eluted into 100 µl of TE buffer and stored at -20°C until further use.

PCR amplification and reverse line blot (RLB) hybridization assay

A total of 120 DNA samples were tested using the RLB hybridization assay as previously described [15,7]. Briefly, the V4 hypervariable region of the parasite 18S rRNA gene was amplified using primers RLB-F2 and RLB-R2 [32], while the V1 region of the parasite 16S rRNA gene was amplified from *Ehrlichia* and *Anaplasma* species using primers Ehr-F and Ehr-R [33]. The PCR reaction was prepared as follows: 5 µl DNA (30–50 ng), 12.5 µl Platinum Quantitative PCR SuperMix-UDG (Invitrogen, The Scientific Group, South Africa), and 20 pmol of each primer made up to a total volume of 25 µl using nuclease-free water. Amplification was done using a touchdown PCR programme as previously described [32]. A *T. parva* positive buffalo DNA sample, 102 [16], and nuclease-free water were used as positive and negative controls, respectively. Amplicons were visualised on a 2% ethidium bromide-stained agarose gel and then screened by the RLB hybridization assay as previously described [15,34]. The *Theileria*, *Ehrlichia*, *Anaplasma* and *Babesia* group- and species-specific oligonucleotide probes used were the same as those described in [35] and [36].

Indirect fluorescent antibody test (IFAT)

After discarding haemolysed samples, only a total of 108 serum samples collected from buffalo were available to be tested using the IFAT [13,37,38] according to OIE standards [39]. The test was conducted at the ARC-OVI using two dilutions, 1/40 and 1/80. The presence of fluorescence in both the 1/40 and 1/80 dilutions was considered as a positive result in serum from buffalo, indicative of the presence of *T. parva* antibodies (Olivier Matthee, personal communication).

T. parva-specific quantitative real-time PCR (qPCR)

Among the 120 smears collected, one sample was discarded because of an insufficient amount of DNA for the test. A total of 119 DNA samples were subjected to the *T. parva*-specific qPCR assay as previously described [16]. Briefly, the *T. parva*-specific forward and *Theileria* genus-specific reverse primers [16] were used to amplify a 167 bp fragment of the parasite V4 variable region of the 18S rRNA gene. For the specific detection of *T. parva* amplicons, the hybridization probes *T. parva* anchor and *T. parva* sensor (640 nm LC Red) were included in the PCR reaction which consisted of 4 µl of 10× LightCycler-FastStart DNA Master^{PLUS} Hybridization Probes mix (with 2× final concentration), 0.5 mM of each primer, 0.1 mM of each hybridization probe, 0.5 U Uracil-deoxy-

glycosylase (UDG) and 4 µl of the template DNA (30–50 ng) with a final volume of 20 µl. A *T. parva* positive buffalo DNA sample, 102 [16], and nuclease-free water were used as positive and negative controls, respectively. Amplification and melting curve analysis were done as previously described [16] in a LightCycler1 v2 (Roche Diagnostics, Mannheim, Germany). Fluorescence values were measured at 640 nm.

Statistical analysis

Descriptive epidemiological measures were analyzed using Epi-Info software (CDC, Atlanta, USA) and were reported as percentages of positive animals to the different diagnostic tests. Chi square test calculations for homogeneity of two populations (Fischer exact test) were used to statistically evaluate the potential influence of age, sex, location and density of buffalo at the capture sites on the observed parasite prevalence. When the variance of the two groups was not homogenous, the Kruskal Wallis test was used. Values of $p < 0.05$ were considered significant. Agreement between the different diagnostic tests assessing the presence of *T. parva* or its antibodies (IFAT, qPCR and RLB) was calculated for those sera having a common result to those tests. Two by two comparisons of the results were expressed using the kappa value. Kappa is a widely used measure of test agreement defined as the quotient of the observed proportion of agreement beyond chance and the maximal proportion of agreement beyond chance [40]. A kappa of 0 indicates no agreement beyond chance, while a kappa of 1 indicates perfect agreement. A kappa of 0.5 indicates a moderate level of agreement.

Ethical statement

The study (Project nr. V082-12) was approved by the University of Pretoria Animal Ethics committee.

Results

RLB

The RLB results (Table 1) indicated the presence of *Theileria*, *Babesia*, *Anaplasma* and *Ehrlichia* species, either as single or as mixed infections in the buffalo populations from two wildlife areas assessed in northern Botswana. From a total of 120 blood smear samples tested, 23 samples (19.2%) contained single infections while 80 (66.7%) contained mixed infections. The most prevalent haemoparasite in the CNP was *T. mutans* (60.9%) followed by *T. parva* (51.6%), *T. buffeli* (46.9%), *B. occultans* (40.6%) and *A. marginale* subsp. *centrale* (31.3%). In the OD, *T. parva* (69.6%) was most prevalent followed by *A. marginale* subsp. *centrale* (28.6%) and *Theileria* sp. (buffalo) (23.2%).

RLB results indicated that a total of 72 of the 120 samples (60.0%) tested positive for *T. parva* DNA. There

Table 1 The occurrence of different haemoparasites in buffalo blood samples from two geographical areas in northern Botswana as determined by the RLB hybridization assay

	Chobe National Park (n = 64)	Okavango delta (n = 56)	Total (n = 120)
Single infections:	5 (7.8%)	18 (32.1%)	23 (19.2%)
<i>T. parva</i>	1 (1.6%)	15 (26.8%)	16 (13.3%)
<i>T. mutans</i>	3 (4.7%)	0	3 (2.5%)
<i>A. marginale</i> subsp. <i>centrale</i>	0	2 (3.6%)	2 (1.7%)
<i>A. marginale</i>	1 (1.6%)	1 (1.8%)	2 (1.7%)
Mixed infections	53 (82.8%)	27 (48.2%)	80 (66.7%)
<i>T. parva</i>	32 (50.0%)	24 (42.9%)	56 (46.6%)
<i>T. mutans</i>	36 (56.3%)	5 (8.9%)	41 (34.2%)
<i>A. marginale</i> subsp. <i>centrale</i>	20 (31.3%)	14(25.0%)	34 (28.3%)
<i>T. buffeli</i>	30 (46.9%)	4 (7.1%)	34 (28.3%)
<i>B. occultans</i>	26 (40.6%)	2 (3.6%)	28 (23.3%)
<i>Theileria</i> sp. (sable)	25 (39.1%)	2 (3.6%)	27 (22.5%)
<i>A. marginale</i>	13 (20.3%)	9 (16.1%)	22 (18.3%)
<i>Theileria</i> sp. (buffalo)	8 (12.5%)	13 (23.2%)	21 (17.5%)
<i>T. velifera</i>	9 (14.1%)	1 (1.8%)	10 (8.3%)
<i>E. ruminantium</i>	4 (6.3%)	3 (5.4%)	7 (5.8%)
<i>T. ovis</i>	3 (4.7%)	1 (1.8%)	4 (3.3%)
<i>Anaplasma</i> sp. Omatjenne	1 (1.6%)	2 (3.6%)	3 (2.5%)
<i>B. bovis</i>	0	2 (3.6%)	2 (1.7%)
<i>Theileria/Babesia</i> genus-specific only	2 (3.1%)	5 (8.9%)	7 (5.8%)
<i>Anaplasma/Ehrlichia</i> genus-specific only	1 (1.6%)	3 (5.4%)	4 (3.3%)
Negative/below detection limit	2 (3.1%)	1 (1.8%)	3 (2.5%)

was a significant difference ($p = 0.042$) in the prevalence of *T. parva*-positive samples between the two wildlife areas. However, there was no significant association between prevalence of *T. parva*, and sex or age of the sampled animals (Table 2).

RLB results also showed significant differences in the prevalence of *T. mutans*, *T. buffeli*, *B. occultans*, *Theileria* sp. (sable) and *T. velifera* infections per wildlife area (Table 3). There was a significant difference ($p \leq 0.05$) between the age of animals that harboured *A. marginale*

Table 2 Comparison of prevalence of *T. parva* per location, age and sex with the three different tests performed

Parameter	RLB	IFAT	qPCR
Wildlife area	CNP 33/64 (51.6%)	39/60 (65.0%)	49/63 (77.8%)
	OD 39/56 (69.6%)	41/48 (85.4%)	47/56 (83.9%)
Age	Young 19/38 (50.0%)	26/36 (72.2%)	27/38 (71.1%)
	Adult 48/77 (62.3%)	54/71 (76.1%)	66/77 (85.7%)
Sex	Male 23/45 (51.1%)	31/43 (72.1%)	37/45 (82.2%)
	Female 44/70 (62.9%)	49/64 (76.6%)	57/70 (81.4%)

Boldfaced values indicate a significant difference between test results for a given parameter ($p \leq 0.05$).

subsp. *centrale*, *T. buffeli*, *B. occultans* and *A. marginale* infections (Table 3). There was a significant association ($p \leq 0.05$) between sex and the buffalo that tested positive for *A. marginale* subsp. *centrale* and *T. velifera* DNA (Table 3).

When comparing the *T. parva* RLB results in the three buffalo capture sites, *T. parva* prevalence was highest in the NG30 samples (76.3%) followed by the MGR (55.6%) samples and the CNP samples (51.6%). Differences in *T. parva* prevalence as determined by RLB were only significant when comparing NG30 versus CNP ($p = 0.01$) (Figure 2). There were no significant differences between the prevalence of other haemoparasites in the different capture sites or the numbers were too small to assess statistical differences

IFAT

T. parva antibodies were detected in 80 of the 108 (74.1%) samples tested (Table 2). Of these, 40 (37.0%) samples tested positive at 1/80 and 40 (37.0%) at 1/40. More buffalo from OD (85.4%) than from CNP (65.0%) were seropositive for *T. parva* (Table 2) and this difference was significant ($p = 0.016$). There was no significant difference between the age and sex of the animals that

Table 3 Comparison of occurrence of other haemoparasites per wildlife area, sex and age as determined by the RLB hybridization assay

	<i>T. mutans</i>	<i>A. marginale</i> ss <i>centrale</i>	<i>T. buffeli</i>	<i>B. occultans</i>	<i>Theileria</i> sp. (sable)	<i>A. marginale</i>	<i>Theileria</i> sp. (buffalo)	<i>T. velifera</i>	<i>E. ruminantium</i>	<i>T. ovis</i>	<i>Anaplasma</i> sp. Omatjenne	<i>B. bovis</i>	
Area	CNP	39/64 (60.9%)	20/64 (31.3%)	30/64 (46.9%)	26/64 (40.6%)	25/64 (39.1%)	14/64 (21.9%)	8/64 (12.5%)	9/64 (14.1%)	4/64 (6.3%)	3/64 (4.7%)	1/64 (1.6%)	0/64 (0.0%)
	OD	5/56 (8.9%)	16/56 (28.6%)	4/56 (7.1%)	2/56 (3.6%)	2/56 (3.6%)	10/56 (17.9%)	13/56 (23.2%)	1/56 (5.4%)	3/56 (5.4%)	1/56 (1.8%)	2/56 (3.6%)	2/56 (3.6%)
Age	Young	16/38 (42.1%)	19/38 (50.0%)	6/38 (15.8%)	4/38 (10.5%)	7/38 (18.4%)	15/38 (39.5%)	4/38 (10.5%)	2/38 (5.3%)	2/38 (5.3%)	2/38 (5.3%)	0/38 (0.0%)	1/38 (2.6%)
	Adult	27/77 (35.1%)	16/77 (20.8%)	27/77 (35.1%)	24/77 (31.2%)	20/77 (26.0%)	8/77 (10.4%)	15/77 (19.5%)	8/77 (10.4%)	5/77 (6.5%)	2/77 (2.6%)	3/77 (3.9%)	0/77 (0.0%)
Sex	Male	21/45 (46.7%)	19/45 (42.2%)	16/45 (35.6%)	14/45 (31.1%)	13/45 (28.9%)	13/45 (28.9%)	10/45 (22.2%)	7/45 (15.6%)	4/45 (8.9%)	3/45 (6.7%)	1/45 (2.2%)	1/45 (2.2%)
	Female	22/70 (31.4%)	16/70 (22.9%)	17/70 (24.3%)	14/70 (20.0%)	14/70 (20.0%)	10/70 (14.3%)	9/70 (12.9%)	3/70 (4.3%)	3/70 (4.3%)	1/70 (1.4%)	2/70 (2.9%)	0/70 (0.0%)

Boldfaced values indicate a significant difference between test results for a given parameter ($p \leq 0.05$).

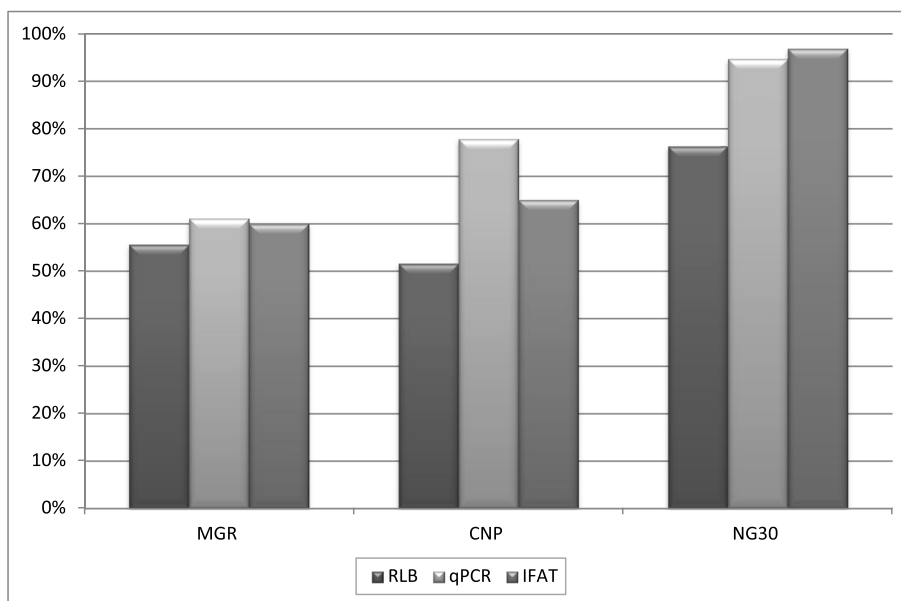


Figure 2 Pair wise comparison of capture location and prevalence of *T. parva* according to the different tests performed. Differences were significant ($p \leq 0.05$) when comparing CNP and NG30 (for the 3 tests) and when comparing MGR and NG30 (for IFAT and qPCR). Note: buffalo densities in those locations were estimated at 1.88 buffalo/km² for the Chobe river, 1.37 buffalo/km² for the MGR and 3.55 buffalo/km² for the NG30 area of the OD.

were seropositive (Table 2). Mean seroprevalence was higher (96.9%) in the NG30 region than in the other two capture sites, 65.0% in the CNP and 60.0% in the MGR and those differences were highly significant. Differences in *T. parva* seroprevalence between MGR and CNP were not significant (Figure 2).

Quantitative real-time PCR (qPCR)

Melting curve analysis (Figure 3) confirmed the presence of *T. parva* DNA in 96 of 119 (80.7%) samples tested. There were no significant differences between prevalence of *T. parva* with this test, and the wildlife area, age or sex of the animals (Table 2), but some significant differences were found when comparing capture sites. Mean prevalence of *T. parva* as determined by qPCR

was higher in the NG30 region (94.7%) than in the other two capture sites; 77.8% in the CNP and 61.1% in the MGR, respectively (Figure 2). The differences in *T. parva* prevalence between MGR and the CNP were not significant but it became highly significant when comparing *T. parva* prevalence in NG30 with that in the other two areas.

Comparison of tests

For IFAT, 80/108 (74.1%) samples tested positive for *T. parva* antibodies, while for the qPCR and the RLB hybridization assay, 96/119 (80.7%) and 72/120 (60.0%) of the samples tested positive, respectively. The Kappa value when comparing IFAT and qPCR indicated a moderate level of agreement (0.561), while the comparison

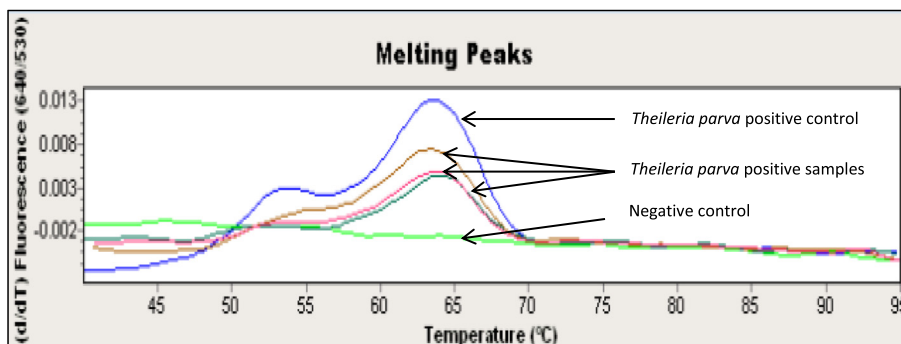


Figure 3 Representative melting curves at $\pm 63^\circ\text{C}$ at 640 nm confirming the presence of *T. parva* positive samples.

with RLB and the two other tests indicated a low level of agreement (Table 4). The observed prevalences of *T. parva* (RLB assay) and *T. parva* antibodies (IFAT) were both significantly higher in the OD than in CNP, while no significant difference was observed for the qPCR test.

Discussion

This is the first report on the presence of tick-borne haemoparasites in African buffalo from two of the most representative wildlife areas from northern Botswana. Our results provide new insights on the distribution of haemoparasites from buffalo transmissible to cattle across the two main wildlife areas in northern Botswana which can be inferred to the distribution of their specific vectors. Several important pathogenic haemoparasites which could present a constraint to the livestock industry in Botswana were identified. These included *T. parva*, *A. marginale*, *B. bovis* and *E. ruminantium*. A higher level of mixed infections was found in CNP compared to the locations sampled in the OD. This may be due to the fact that the Chobe river is the main source of water in the CNP and a high number of buffalo herds congregate along this water source during the dry season which might facilitate the exchange of ticks and their parasites between different individuals. In the OD, there is water all year round and despite some animal densities might be locally higher, buffalo herds might have less interactions between each other.

The RLB, IFAT and qPCR tests all indicated a high prevalence of *T. parva* presence or exposure in both CNP and OD. This indicates a high risk of spreading Corridor disease caused by *T. parva* from buffalo to cattle by the vector ticks at the livestock-wildlife interface. Other haemoparasites with high prevalence identified by the RLB included *T. mutans*, *T. buffeli*, *B. occultans* and *Theileria* sp. (sable) in CNP and *A. marginale* subsp. *centrale*, *Theileria* sp. (buffalo) and *A. marginale* in OD. Generally speaking, the buffalo population in the OD sample had lower levels of haemoparasite infections than the one in the CNP sample, with the exception of *Theileria* sp. (buffalo) and to a lesser extent *Anaplasma* sp. Omatjenne and *B. bovis* (in the two later cases, with very few positives were detected). In the specific case of *T. parva*, a significant association was observed between densities of buffalo in the capture location of the herds (only 3 measures of density were available) and prevalence

found with the different tests, particularly in the case of the IFAT and qPCR results. This was more evident when comparing those areas where differences between density figures were more extreme (cf MGR and the NG30). However, buffalo density is only one possible cause of those differences, and many other habitat variations or ecological factors affecting host health or vector distribution and density in the different range areas where the buffalo herds were sampled, could also be responsible for those differences [41,42]. Therefore, further studies with a higher number of data and measures at the different herd locations would be necessary to detect explanatory factors accounting for those prevalence differences.

T. parva antibodies were detected in 74.1% of samples tested using the IFAT. Limitations of the IFAT include standardization of the test in buffalo samples, subjectivity towards the interpretation of results acquired and the difficulty of detecting low levels of parasite antibodies [9,43]. The IFAT is highly sensitive when testing for antibodies for only one species of *Theileria*, but in areas where different species overlap; cross-reactions between *Theileria* species are common (especially between *T. parva*, *T. annulata* and *T. taurotragi*) and reduce the specificity of the test [9,40]. However, the geographical distribution of *T. annulata* and *T. parva* does not overlap [44] and *T. taurotragi* was not identified in the Botswana buffalo samples. Therefore, the likelihood of cross-reactivity can be ruled out and our results most likely reflect the real presence and burden of *T. parva*, which would explain the high level of agreement between IFAT and qPCR. Another factor to take into account is the period elapsed from infection and the development of antibodies. With the use of schizont antigen, *T. parva* antibodies can first be detected 10 to 14 days post-infection and with piroplasm antigens 15 to 21 days post-infection. High levels of antibodies are still detectable 30 to 60 days after the animals have recovered from a *T. parva* infection which is followed by the gradual decrease of antibody levels. Antibodies can still be detected 4 to 6 months post-recovery and may persist for up to a year at such low levels that they may not be detected at a serum dilution of 1/40 [39]. The animals in our study may have carried *T. parva* infections for a very long time as suggested by the very low antibody levels observed.

Comparing the efficiency of the different tests, we found that the qPCR (80.7%) and IFAT (74.1%) were far better in identifying *T. parva* positive samples than the RLB assay (60.0%). Although there was correlation between the qPCR and RLB results in the detection of *T. parva*, minor differences between the results were observed; most notably in those samples with mixed haemoparasite infection. The qPCR assay can reliably detect *T. parva* in carrier animals with a piroplasm parasitaemia as

Table 4 Agreement expressed by kappa value when comparing diagnostic tests for *Theileria parva* two by two

Tests compared	Sample size	Kappa value	Standard error 95% CI
RLB vs qPCR	119	0.256	0.09 [0.09;0.472]
IFAT vs qPCR	107	0.561	0.096 [0.3;0.7]
IFAT vs RLB	107	0.154	0.094 [-0.031;0.3]

low as $8.79 \times 10^{-4}\%$ [16]. The sensitivity of the RLB assay was determined at $10^{-6}\%$ parasitaemia, by testing serial dilutions of *T. annulata*-infected blood samples [15]. However, due to the likely presence of multiple parasites present in one sample, competition for available primers may occur in the PCR which in turn would lead to an underrepresentation of some of the parasites detected by RLB alone. Furthermore, weak hybridization and/or cross-reactivity of probes may cause the RLB hybridization assay to yield less sensitive results than the qPCR and IFAT [45]. In addition, mixed infections could mask the presence of novel genotypes in the RLB assay and other tests would be needed to identify them [46,47,15].

Risk factors influencing the prevalence of tick-borne parasites may include the distribution of tick vectors, the abundance of buffalo and cattle and their movement/migratory patterns, resistance of the hosts to the parasites and their tick vectors [48] and age of the host [42]. It has previously been found that older animals have a higher tick load than younger ones [42]. However, higher tick loads do not necessarily mean higher infection rates of haemoparasites. A model designed in East-Africa also determined that in wildlife-livestock interfaces where only cattle were treated with acaricides, *T. parva* remained a problem because this treatment had no effect on the disease transmission in buffalo. In addition, the continuous use of acaricides can have significant economic and ecological consequences [49]. Our study only identified a significant difference between age and infection rate for *B. occultans* and *Theileria* sp. (sable), which depending on the species of parasite, were higher in young (*A. marginale*, *A. marginale* subsp. *centrale*,) or in adult animals (*B. occultans* and *Theileria buffeli*). However, our sample was too small to be able to detect a consistent trend between age and infestation.

A number of studies have previously been conducted on buffalo in South Africa. In the Marakele National Park (MNP) and Kruger National Park (KNP) buffalo were tested for the presence of *Theileria* spp. using the RLB hybridization assay and the *T. parva*-specific qPCR assay [50]. The RLB results indicated the presence of *T. parva*, *Theileria* sp. (buffalo), *T. mutans*, *T. buffeli* and *T. velifera* in both parks. The qPCR assay identified 70% of samples positive for *T. parva* and the RLB results indicated 40% of samples in both parks. In a separate study in the Hluhluwe-iMfolozi Park and the Greater Limpopo Transfrontier Park, *T. parva*, *Theileria* sp. (buffalo), *T. mutans*, *T. buffeli* and *T. velifera* were also identified in African buffalo [7]. In both studies, the *T. parva*-specific qPCR was found to be more sensitive than the RLB, correlating with the results found in our study. In another study done in the Hluhluwe-iMfolozi Park and Kruger National Park, Debiela [51] found the same *Theileria*

spp. as in our study in Botswana, as well as *A. marginale* subsp. *centrale*, *A. marginale*, *Anaplasma* sp. Omatjenne, *E. ruminantium* and *B. occultans*.

In East Africa, in a study done in four different national parks in Uganda, buffalo were found to be carriers of *T. parva*, *T. mutans*, *T. velifera*, *A. marginale* and *A. marginale* subsp. *centrale* [52]. In two of these parks, buffalo also carried *T. buffeli* and *Theileria* sp. (buffalo). None of the animals sampled were carriers of *T. taurotragi*, *B. bovis*, *B. bigemina*, *A. bovis* or *E. ruminantium*. As in Uganda, the pathogenic *B. bovis* has previously been reported to be absent from buffalo in Botswana [53]. However, in the current study, we identified the parasite to be present in a low percentage of the OD buffalo tested. Similarly, *E. ruminantium* could be identified in a few CNP and OD buffalo tested. The significance of buffalo as possible reservoir host of some of these economically important haemoparasites (i.e. *A. marginale*, *E. ruminantium*) remains unknown.

Theileria sp. (sable), which is fatal to sable (*Hippotragus niger*) and roan antelope (*Hippotragus equinus*), but non-pathogenic to buffalo [34] was identified in some of the Botswana buffalo. However, it should be noted that the positive RLB signals might be due to cross reactions of the *Theileria* sp. (sable) probe with genotypes similar to *Theileria* sp. (sable) and/or with *T. velifera* and should be interpreted with caution [12]. Similarly, four samples tested positive for *T. ovis* which is usually found in goats and sheep. We can only speculate whether these are true findings due to incidental infections, or whether they are as a result of cross-reaction of the RLB probes with previously unknown targets or contamination with other target DNA.

The following important tick vectors have been identified in Botswana in previous studies: *Amblyomma variegatum*, *Rhipicephalus decoloratus*, *R. zambeziensis*, *R. evertsi evertsi*, *R. simus*, *Hyalomma truncatum* and *H. marginatum rufipes* [54-56]. These ticks are known to transmit most of the haemoparasites found in this study. These vectors may also be responsible for the transmission of *T. buffeli* and *Theileria* sp. (buffalo) but further research is needed to confirm this hypothesis, since the tick vectors of these parasite species remain unknown.

T. parva is known to occur in Zambia [8] and South Africa [57,58], but this is the first written report of its occurrence in northern Botswana, despite its presence has been suspected for several years. Currently, there are no regulations instituted for the systematic surveillance and control of tick-borne diseases in Botswana. In addition, Corridor Disease in cattle is fulminant and makes it difficult to detect clinical cases in live animals. Therefore, the present work emphasizes the role of the African buffalo, as a sentinel species to identify the presence and circulation of livestock pathogens. The

presence of *Rhipicephalus appendiculatus* and East Coast fever in northern Botswana has been predicted in spatial risk models by some authors [59]. When infected buffalo share the same home ranges with cattle and other domestic animals, those haemoparasites can be transmitted to cattle through infected tick bites. This information on the circulation of TBD can contribute to raise awareness among veterinary officials and rural communities living at the wildlife-livestock interface so that control measures (prevention of wildlife-cattle contacts, regular dipping) can be implemented to mitigate their economic impact. In a recent comparative assessment of cattle herds in three different wildlife/livestock interfaces in Zimbabwe, significantly higher levels of *T. parva* antibodies were found in those areas that were unfenced when compared with those that had a physical separation between wildlife and livestock [60]. Therefore, we can hypothesize that this parasite is less likely to be transmitted from buffalo to cattle in the OD, due to the presence of a veterinary cordon fence preventing contacts with cattle and surrounding the game reserve. To the contrary in the CNP, where there is no physical separation between buffalo and cattle, transmission of common diseases from buffalo to cattle is likely to occur more frequently [29] and future surveillance efforts should be targeted in priority towards livestock from this area.

Conclusions

This paper illustrates the diversity of haemoparasites present in African buffalo from northern Botswana and highlights the role of African buffalo as a sentinel species for livestock tick-borne pathogens. Our results indicate the significance of the African buffalo as reservoir host for important tick-borne haemoparasites that can cause severe disease in cattle. They also suggest that qPCR and IFAT are more efficient in detecting *T. parva* exposed buffalo than the RLB test. These results should contribute to raise awareness among veterinary authorities regarding the potential occurrence of these parasites in cattle so that appropriate control and surveillance protocols taking into account the presence of infected wildlife reservoirs in those areas can be designed at the wildlife-livestock interface.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

DE carried out the molecular diagnostic assays and wrote the first draft of the manuscript. FJ conducted fieldwork for the collection of blood, performed the statistical analysis and interpreted the data. KPS provided qPCR training, while the training in DNA extraction and the RLB hybridization assay were provided by IV and MT. MCO supervised the laboratory work, interpreted the data and is the main supervisor of the student (DE). DE, FJ, NE and MCO arranged the data, formulated and prepared the manuscript. All authors read and approved the final manuscript.

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APPENDIX II



UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

PROJECT TITLE	The characterization of <i>Theileria parva</i> in buffalo (<i>Syncerus caffer</i>) from Botswana using the p67, p104 and poly-morphic immunodominant molecule (PIM) genes
PROJECT NUMBER	V082-12
RESEARCHER/PRINCIPAL INVESTIGATOR	Mr. D Eygelaar

STUDENT NUMBER (where applicable)	26029104
DISSERTATION/THESIS SUBMITTED FOR	MSc

ANIMAL SPECIES	Buffalo (<i>Syncerus caffer</i>)	
NUMBER OF ANIMALS	121	
Approval period to use animals for research/testing purposes		February 2012 – December 2013
SUPERVISOR	Prof. M Oosthuizen	

KINDLY NOTE:

Should there be a change in the species or number of animal/s required, or the experimental procedure/s - please submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experiment

APPROVED	Date	29 April 2013
CHAIRMAN: UP Animal Ethics Committee	Signature	

TELEPHONE: 3971405
FAX: 3912354
TELEGRAMS: GAME GABORONE
REFERENCE:



REPUBLIC OF BOTSWANA

DIRECTOR OF WILDLIFE & NATIONAL PARKS
P.O. Box 131
GABORONE

29th Mǎy 2013

WP/RES 15/2/2 XXII

PLEASE ADDRESS ALL OFFICIAL COMMUNICATION TO THE DIRECTOR

Dewald Eygelaar
University of Pretoria
South Africa

Dear Sir,

Request for permission to use samples from the NIDI project

Your correspondence dated 20th May 2013 refers.

You are hereby granted permission to use the samples (GIEMSA-stained bloodsmear slides) from the Ngamiland Interface Disease Investigation (NIDI) project. It is noted that the samples shall be used for identifying *Theileria parva* as part of an MSc. project.

We would greatly appreciate a copy of your thesis.

Your faithfully,

A handwritten signature in black ink, appearing to read 'M. Flyman'.

Dr Michael Flyman
Chief Wildlife Officer (Research and Statistics)





agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

Private Bag X138, Pretoria, 0001
Delpen Building, c/o Annie Botha & Union Street,
Riviera, 0084

From: Directorate Animal Health
Tel: +27 12 319 7507
Fax: +27 12 329 6892
E-mail: LeanaJvR@daff.gov.za
Enquiries: Dr Leana Janse van Rensburg

Ref: 13/1/1/30/2/10/2

13 June 2011

Ferran Jori
Mammal Research Institute
Department of Zoology and Entomology
University of Pretoria
South Africa

Re: Movement of samples from the OVI to the University of Pretoria.

Hereby permission is granted to move extracted DNA samples obtained from buffalo from Northern Botswana that have been sufficiently processed to inactivate Foot and Mouth Disease virus, confirmed in writing, from the Onderstepoort Veterinary Institute to the Department of Zoology to be tested under the supervision of Dr Armanda Bastos and to the Department of Veterinary Tropical Diseases to be tested under the supervision of Dr Marinda Oosthuizen..

Kind regards,

A Schoeman
For Director: Animal Health

All communications should be addressed to
"CVRO (Diagnostics and Research)"

Telephone: 263-4-705885/6/7
Telegram: "VACCINE" HARARE
Telefax: 263-4-707952
Email: vetlabs@africaonline.co.zw
Website: www.vetdiaphres.org.zw



ZIMBABWE

Reference:

CENTRAL VETERINARY LABORATORY
Laboratory Diagnostics and Research Branch
Department of Veterinary Technical Services
P O Box CY 551, Causeway
Harare

University of Pretoria
Department of Veterinary Tropical Diseases
Old Soutpan road
Paraclinical Building 2-11
Onderstepoort 0110
South Africa

RLB 82/12 - RLB 121/12.

0110

Tel: (012) 3177514

October 11 2010

To Diagnostic services

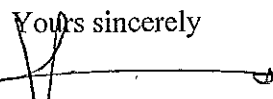
REF.: Molecular biology typing

This letter serves to confirm that this consignment contains 30 Blood samples on filter paper from Central Veterinary laboratory for Molecular Biology typing tests, for the purposes of interlaboratory testing. The samples are non infectious

For any queries and clarifications you can contact the above numbers. Thank you in advance for your cooperation.

The master import permit number is 13/1/1/30/4-85

Yours sincerely


Dr. C. Gomo

DIRECTOR OF VETERINARY
SERVICES

12 OCT 2010

P.O. BOX CY 551, CAUSEWAY
HARARE

Samples originate from Gonarezhou National Park.
Zimbabwe.

For Maingda
(Int)