ASSESSMENT AND IMPROVEMENT OF MOLECULAR DIAGNOSIS OF THEILERIA PARVA OF AFRICAN BUFFALO (SYNCERUS CAFFER) IN SOUTHERN AFRICA

By

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DECLARATION

I declare that this dissertation hereby submitted for the degree of Magister Scientiae, Zoology at the University of the Free State is the original work by the author under the supervision of Prof. Barend J. Mans and Dr. Oriel M.M. Thekisoe. The dissertation has not been submitted in any form to another University. I therefore cede copyright of this dissertation in favour of the University of the Free State.

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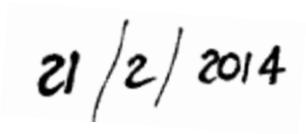

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ABSTRACT

Buffalo-adapted *Theileria parva* causes Corridor disease in cattle. Strict control measures therefore apply to the movement of buffalo in South Africa and include mandatory testing of buffalo for the presence of *T. parva*. The official test is a real-time hybridization PCR assay that amplifies the V4 hypervariable region of the 18S rRNA gene of *T. parva, T.* sp. (buffalo) and *T.* sp. (bougasvlei). The effect that mixed *T. parva* and *T.* sp. (buffalo)-like infections have on accurate *T. parva* diagnosis was investigated. *In-vitro* mixed infection simulations indicated PCR signal suppression at 100 to 1000-fold *T.* sp. (buffalo) excess at low *T. parva* parasitaemia. Suppression of PCR signal was found in field buffalo with mixed infections. The *T. parva*-positive status of these cases was confirmed by selective suppression of *T.* sp. (buffalo) amplification using a locked nucleic acid clamp and independent assays based on the p67, p104 and *Tpr* genes. Conventional and SYBR® Green touch-down PCR methods were developed for each protein coding gene and buffalo from the endemic Kruger National Park were screened. The protein gene assays compared well with the negative and *T. parva* positive samples diagnosed on the current real-time assay however, they did detect additional positive samples diagnosed as negative on the real-time hybridization. These samples were all *T.* sp. (buffalo) positive. This confirmed the suppressive effect on PCR signal due to template competition in the current real-time PCR assay. Some positive samples were not detected by the protein genes, possibly due to sequence variation in the primer regions. These independent markers proved useful as supplementary assays in the accurate diagnosis of *T. parva* infections where mixed infections occur in the buffalo host. The development of the Hybrid II assay, a real-time hybridization PCR method, which compared well with the official hybridization assay in terms of specificity and sensitivity revolutionized the diagnosis of the disease for the main reason that it is not influenced by mixed infections of *T.* sp. (buffalo)-like parasites and is as such a significant improvement on the current hybridization assay. While the incidence of mixed infections in the Corridor disease endemic region of South Africa is significant, little information is available on the specific distribution and prevalence of *T.* sp. (buffalo) and *T.* sp. (bougasvlei). Specific real-time PCR assays were developed and a total of 1211 samples known to harbor these parasites were screened. Both parasites are widely distributed in southern Africa and the incidence of mixed infections with *T. parva* within the endemic region is similar (~25-50%). However, a significant discrepancy exists with regard to mixed infections of *T.* sp. (buffalo) and *T.* sp. (bougasvlei) (~10%). Evidence for speciation between *T.* sp. (buffalo) and *T.* sp. (bougasvlei) is supported by phylogenetic analysis of the COI gene, and their designation as different species. This suggests mutual exclusion of parasites and the possibility of hybrid sterility in cases of mixed infections.

Keywords: Corridor disease, mixed infection, Real-time PCR, *Theileria parva*, *Theileria* sp. (buffalo), *Theileria* sp. (bougasvlei), speciation, *Syncerus caffer*

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CHAPTER 1

Introduction to Theileria parva

1.1 Classification and Nomenclature of *Theileria*

The causative agent of Corridor disease (CD), East Coast fever (ECF) and January disease (Zimbabwe theileriosis), *Theileria parva,* is classified as an Apicomplexan parasite (Levine et al. 1980)(Table 1.1). The phylum Apicomplexa makes up a large group of complex eukaryotic organisms known to be obligate parasites of vertebrates and invertebrates. Depending on their host niche environments and the stage of lifecycle they are reasonably host specific and exhibit a variety of morphological shapes. Furthermore, the phylum is divided into three principal groups; Coccidia, comprising of the haemogregarines and coccidia, Gregarina (gregarines) and Hematozoa which includes the malarial parasites and piroplasms (Fig.1.1) (Šlapeta and Morin-Adeline, 2011).

Table 1.1: Scientific classification of the species *Theileria parva*, *T.* sp. (buffalo) and *T.* sp. (bougasvlei).

Scientific classification:	
Domain	Eukaryota
Kingdom	Chromalveolata
Phylum	Apicomplexa
Class	Aconoidasida
Order	Piroplasmida
Family	Theileriidae
Genus	*Theileria*
Species	*T. parva*, *T.* sp. (buffalo), *T.* sp. (bougasvlei)

The order Piroplasmida falls under the class Aconoidasida in which the two genera(*Babesia* and *Theileria*) group which cause most of the economic important diseases of domestic and wild animals. These parasites are mainly transmitted by ticks which are renowned for the large economic losses they cause to the agricultural industry due to disease outbreaks, mortalities, damage to hides and poor production to name but a few. The genus of importance in this study is *Theileria*, which has distinct geographic distributions determined by their tick vectors (Table 1.2). The focus will be the species *Theileria parva*, the causative agent of Corridor disease in

cattle, as well as *T.* sp. (buffalo) and *T.* sp. (bougasvlei). The latter two are of lesser economic importance but affects the diagnostic design of molecular assays for *T. parva*.

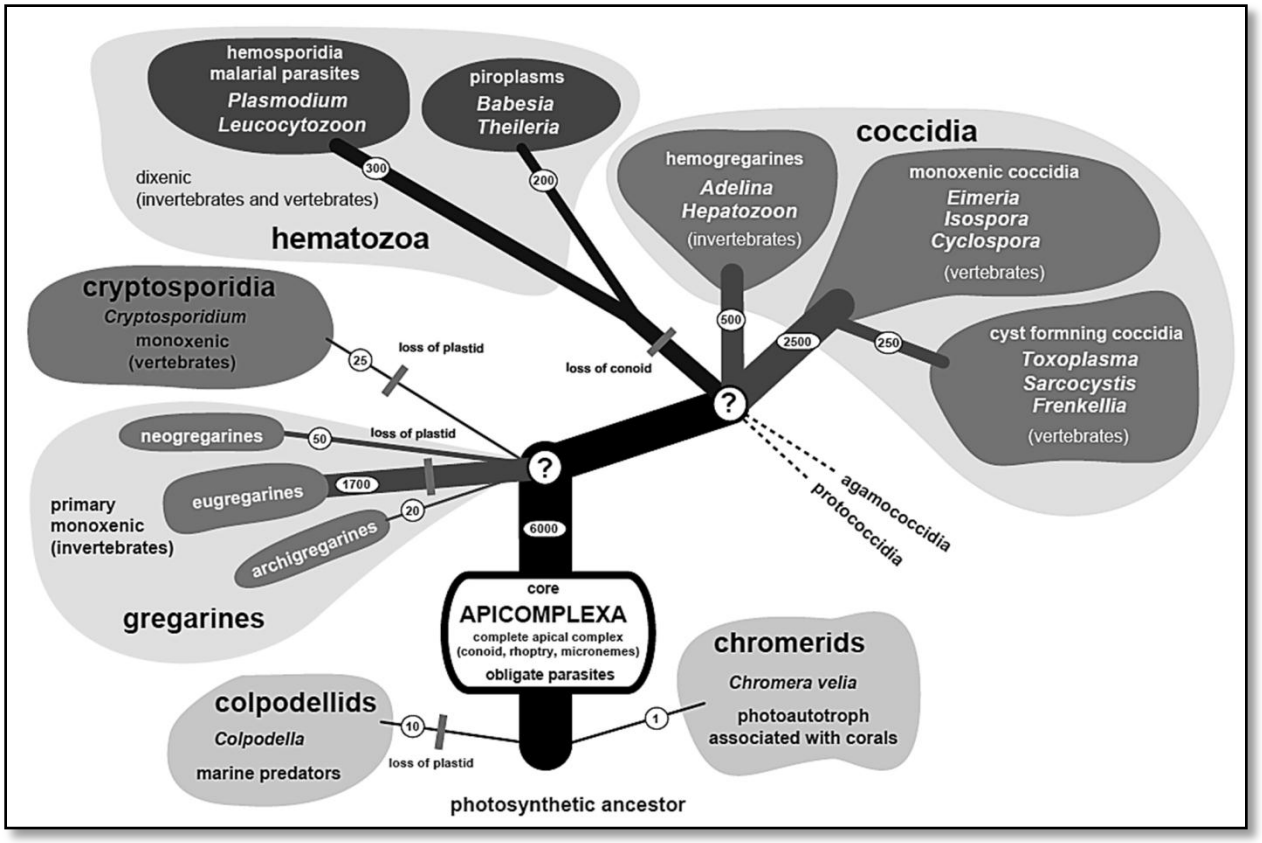


Figure 1.1:A hypothetical tree of Apicomplexa depicting different parasitic groups. Numbers on the branches and thickness indicates diversity (i.e. named species.) (Šlapeta & Morin-Adeline, 2011).

Historically *T. parva* was grouped into three 'subspecies' based on clinical disease and demonstration of the parasite in the vertebrate host (Uilenberg, 1976). *Theileria parva* group consisted of *Theileria parva parva*, *Theileria parva lawrencei* and *Theileria parva bovis*.*Theileria parva parva* was transmitted from cattle to cattle, causing classical East Coast fever (ECF) with mortalities of ~90% of infected hosts (Lawrence, 1979). *Theileria parva lawrencei* was transmitted from infected buffalo to cattle causing Corridor disease (CD) with few piroplasms and schizonts and slaying its host before a carrier state could be reached (Norval et al. 1991), while *T. p. bovis* was a milder disease than ECF or CD and never considered to be present in South Africa (Neitz, 1957). The three species were later combined into *T.*

*parva*because of the lack of a clear biological basis for a separate classification system (Allsopp et al. 1989, Conrad et al. 1989).

Table 1.2:*Theileria* species infective to domestic ruminants, their tick vectors and geographical distribution (Bishop et al. 2008).

***Theileria* species**	**Major vectors**	**Distribution range**
Theileria parva	*Rhipicephalus appendiculatus* *R. zambeziensis* *R. duttoni*	Eastern, central and southern Africa
Theileria annulata	*Hyalomma anatolicum* and other *Hyalomma* spp.	Southern Europe, western, southern and eastern Asia, northern Africa
Theileria mutans	*Amblyomma variegatum* and four other *Amblyomma* spp.	Western, eastern, central and southern Africa, Caribbean islands
Theileria velifera	*Amblyomma variegatum* and other *Amblyomma* spp	Western, eastern, central and southern Africa
Theileria taurotragi	*R. appendiculatus* *R. zambeziensis* *R. pulchellus*	Eastern, central and southern Africa
Theileria sergenti	*Haemaphysalis* spp.	Japan, Korea
Theileria buffeli	*Haemaphysalis* spp.	Europe, Asia, Australia, eastern Africa
Theileria lestoquardi	*Hyalomma* spp.	Asia, northern Africa
Theileria ovis	*Hyalomma* spp.	Asia
Theileria separata	*Hyalomma* spp.	Asia

1.2 Lifecycle of *Theileria parva*

Theileria parva portrays a lifecycle typical of the *Theileria* genus where infective sporozoites enter lymphoid cells of the host where they proliferate through schizogeny and release

merozoites after merogony to invade red blood cells (Fig. 1.2). This leads to the clinical manifestation of the disease and lymphoproliferative pathology associated with high mortality rates among cattle (Neitz, 1955, Norval et al., 1992). The vectors of *T. parva* found in South Africa; *Rhipicephalus appendiculatus* and *Rhipicephalus zambeziensis*, become infected as nymphs, after ingestion of infected erythrocytes from an infected buffalo host (Fig. 1.3) (Neitz, 1955, Lawrence et al. 1983). The parasite undergoes syngamy in the tick gut, forming a zygote which later divides into motile kinetes that infects the tick gut epithelial cells and migrate to the hemolymph. Subsequently, kinetes infect the acini of the salivary glands of the adult tick where they undergo sporogony during the slow feeding phase of the ixodid tick. The sporozoites are released via the saliva of the tick into the laceration created in the dermis of the mammalian host (Norval et al. 1992).

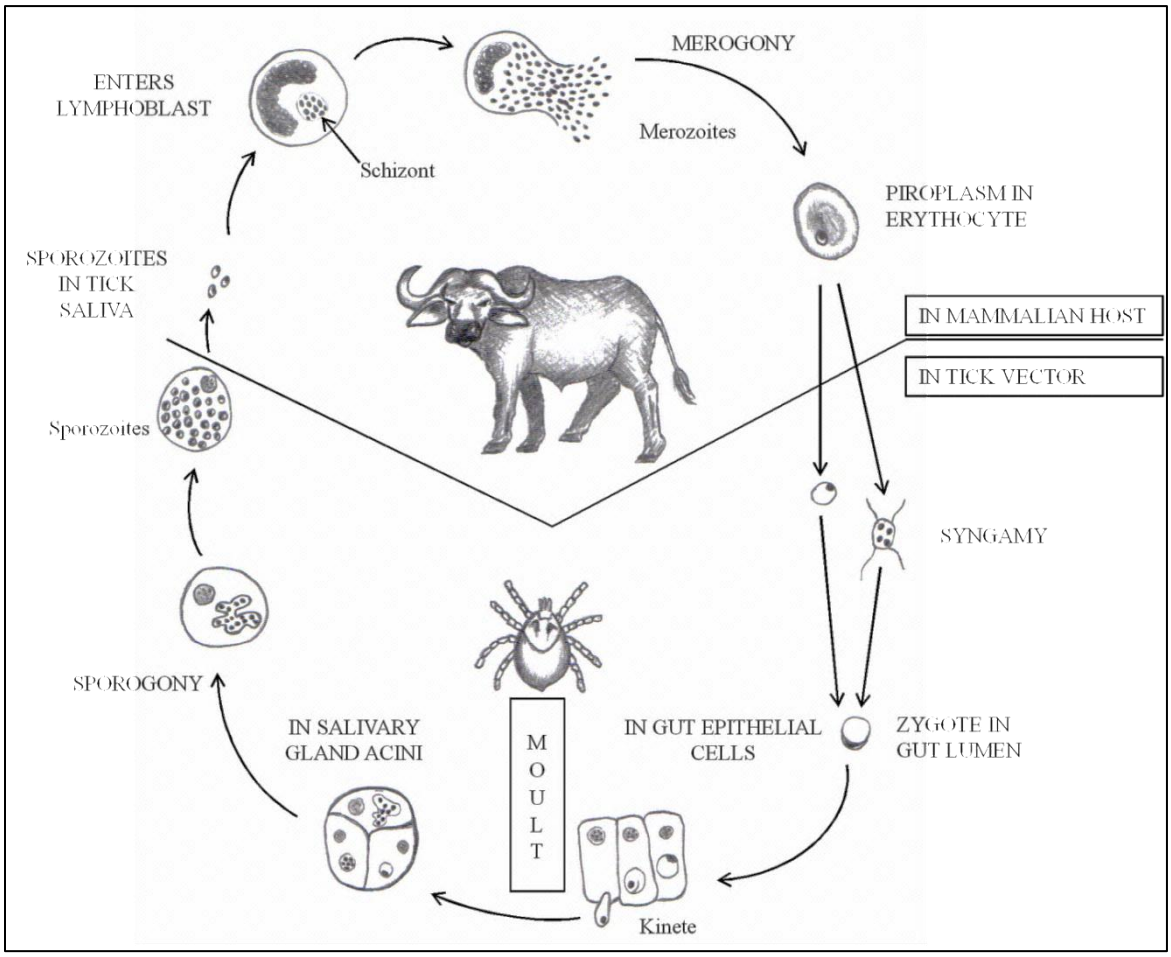


Figure 1.2: Lifecycle of *Theileria parva* in the mammalian host and tick vector.

1.3 **History of East Coast Fever and Corridor disease in South Africa**

ECF was introduced into South Africa from East Africa causing massive morbidity and mortality rates in cattle (Theiler, 1904). The disease was supported and spread because of the presence of the vector tick (Fig. 1.3) but by1956, after an extensive tick control, strict quarantine, systematic dipping and slaughtering campaign, the disease was eradicated (Neitz, 1957). To date, ECF is considered to be absent in South Africa but is still present in East and southern Africa (Potgieter et al. 1988; Sibeko et al. 2008). Corridor and January disease were first identified in Zimbabwe, however, January disease was never considered present in South Africa (Neitz, 1957; Potgieter et al. 1988) while Corridor disease is regarded as endemic to South Africa (Laubscher et al. 2012). The first incident of Corridor disease in South Africa was recorded in the corridor between the historical Hluhluwe and Umfolozi game parks during a tsetse fly eradication campaign leading to great losses in cattle with symptoms similar to 'Buffalo disease' (January disease) in Zimbabwe (Neitz, 1955).

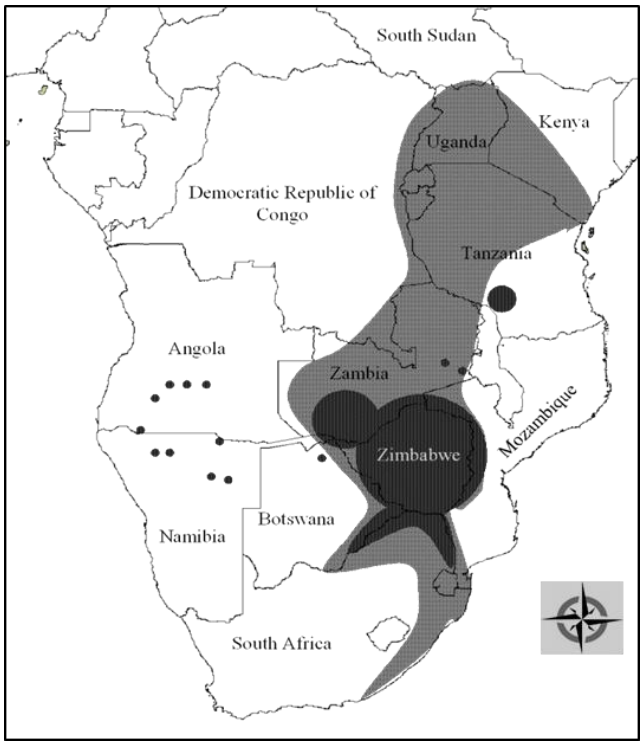


Figure 1.3: Map of southern Africa depicting the distribution of *R. appendiculatus* from East Africa to South Africa (grey shaded area). *R. zambeziensis* have a more limited and dispersed distribution (darker shaded area) (Walker et al. 2000).

ECF and January disease differ from Corridor disease in its transmission mode. The latter is transmitted from carrier African buffalo (*Syncerus caffer*) to cattle while ECF and January disease are transmitted from carrier cattle to other susceptible cattle (Norval et al. 1991). The carrier state in cattle has not been confirmed under field conditions in South Africa yet (Neitz, 1958; Barnett and Brocklesby, 1966a; Potgieter et al. 1988; Mbizeni et al. 2013).

Endemic regions for Corridor disease in South Africa are the Kruger National Park (KNP) in Mpumalanga and the Hluhluwe-Umfolozi Park in KwaZulu-Natal with neighboring areas (Potgieteret al. 1988) (Fig 1.4). The distribution of *T. parva* parasites is dependent on the geographical distribution of infective hosts and the presence of the vector ticks (Fig.1.4).

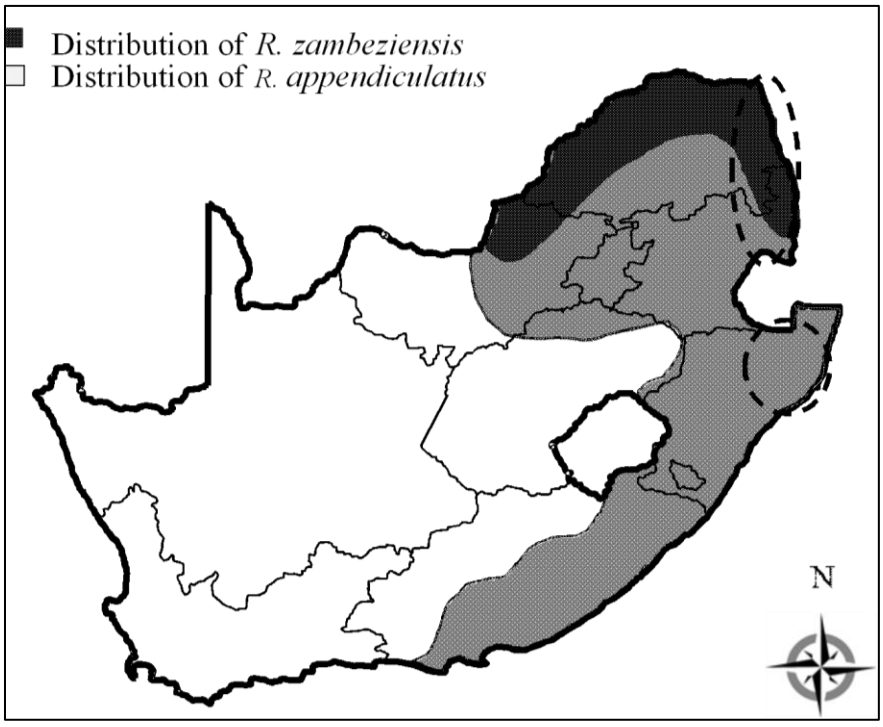


Figure 1.4: Map of South Africa depicting the endemic regions of Corridor disease with Kruger National Park the more northern encircled area and the Hluhluwe-Umfolozi Park more to the south in relation to the distribution of the vector ticks.

If the distribution of its vector ticks, *R. appendiculatus* and *R. zambeziensis*, with the potential movement of carrier buffalo is considered, the possibility to extend into non-endemic areas in the North-West and Northern Province as well as Mpumalanga, Kwa-Zulu Natal and the eastern parts of the Eastern Capeexist (Estrada-Peña, 2003). The possibility of this risk increased

in recent years as the wildlife industry in South Africa underwent a radical change with the development of new game ranches and the expansion of existing private reserves through the re-stocking of previously cattle grazed veld with various species of game. The revolution in the buffalo industry came in 1998, with the inception of ‘disease free’ buffalo breeding projects and the formation of a Buffalo Advisory Committee to control ‘disease-free’ buffalo breeding projects through the registration of breeding projects and properties as well as the monitoring of movement of animals between endemic and non-endemic areas (Laubscher et al. 2012).Buffalo became one of the most sought-after commodities for introduction into these areas since they form part of Africa’s charismatic 'Big 5'. As constraint and small safeguard before translocation of buffalo is granted, compulsory testing for Foot and mouth, Corridor disease and tuberculosis is mandatory to certify buffalo as disease free. These are controlled and notifiable animal diseases in South Africa which are regulated by the Department of Agriculture, Forestry and Fisheries as stipulated by the Animal Disease Act 1984, Act No. 35. The translocation of buffalo is thereforeregulated between and within endemic and non-endemic regions. Currently, all preventive measures have to be taken to prevent the possible establishment of a carrier state of buffalo-derived *T. parva* infections in recovered cattle (Potgieter et al. 1988). However, intermittent Corridor disease outbreaks do occur and have serious implications to the livestock trade industry due to mortalities in naive cattle found in areas where the vector ticks occur in the presence of carrier buffalo (Mbizeni et al. 2013).

1.4 Genetically related *Theileria* species

It was demonstrated in a previous study that the genotypic diversity in buffalowith regard to amount of genotypes and number of co-infecting genotypes are quite extensive (Mans et al. 2011a).Five evolutionary clades were identified when *Theileria* species found in buffalo were phylogeneticaly analysed (Fig 1.5). *Theilera parva* grouped with *T*. sp. (buffalo) and *T*. sp. (bougasvlei) in the *T. taurotragi* clade. Other genotypes found in co-infection in buffalo were from the *T. mutans, T. velifera* and *T. buffeli* clades.

T. sp. (buffalo) was first identified in buffalo from East Africa and distinguished from *T. parva* based on antigenic differences (Conrad et al. 1987).The differences were confirmed by

subsequent sequencing of its 18S ribosomal RNA (rRNA) gene (Allsopp et al. 1993). Its close resemblance to *T. parva* in the 18S rRNA gene region raised the question whether or not this was a variant strain of*T. parva* (Conrad et al. 1987; Allsopp et al. 1993). Soon thereafter the parasite was detected in 54% of animals tested from the Kruger National Park, establishing its presence in South Africa (Allsopp et al. 1999). Successful establishment of a *T.* sp. (buffalo) infected cell line from a buffalo of South African origin demonstrated that on 18S rRNA level, it was identical to the parasite described from East Africa (Zweygarth et al. 2009).

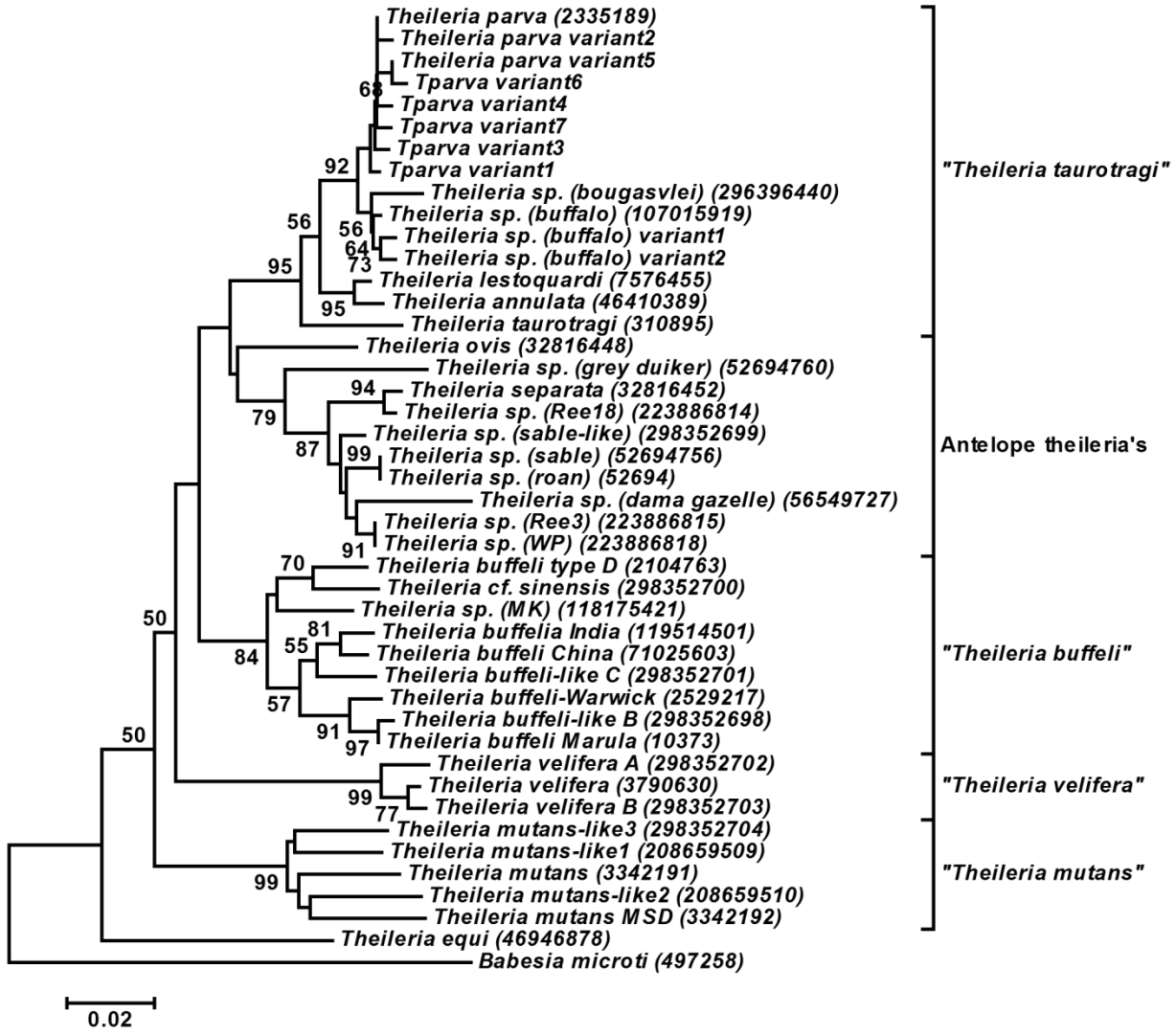


Figure 1.5: Phylogenetic analysis and genotypic variants of *Theileria* species found in buffalo. Species are followed by their GenBank Accession numbers. The tree was rooted using *Babesia microti*asoutgroup. Bootstrap support above 50% is indicated.

T. sp. (bougasvlei) is another closely related parasite recently described (Chaisi et al. 2011; Mans et al. 2011a; Zweygarth et al. 2009).However, little is known about its biology and origins, except that it differs from *T.* sp. (buffalo) at three positions in the hyper-variable region of the 18S rRNA gene (Mans et al. 2011a), and at 8-10 positions across 1505 bp of the whole 18S rRNA gene (Chaisi et al. 2011).These two species are discussed in more detail in Chapter 5.

1.5 Diagnosis of *Theileria parva*

The diagnosis of *T. parva*carrier buffalo evolved from using light microscopy and the indirect immuno-fluorescent antibody test (IFAT) to various molecular based tests involving the amplification of the18S rRNA gene by means of a set of universal *Theileria* genus primers and a slot blotting hybridization technique to real-time polymerase chain reactions (PCRs) (Burridge and Kimber 1972; Potgieter et al. 1988; Allsopp et al. 1993; Collins et al. 2002; Papli et al. 2011; Pienaaret al. 2011b and PVVD diagnostic archival material). The IFAT was initially used during the mass screening of animals for *T. parva* infections. The limitation of the IFAT was cross reactions with other *Theileria* parasites and the lack in sensitivity in the case of carrier animals whose antibody titers waned over time. Light microscopy was also performed on Giemsa stained blood smears (PVVD diagnostic archival material), however, the detection of carrier animals regularly went unnoticed upon smear examination and the discrimination between piroplasms of other *Theileria* species was difficult as they are morphologically very similar (Lawrence, 1979). A vast improvement on the detection of carrier animals was achieved with the use of PCR detection assays targeting various molecular markers (Bishop et al. 1992), however, a drawback was still the use of agarose gels and radio-isotope labeled probes in the post-amplification step of slot-blotting and hybridization (Allsopp et al. 1993).The development of the reverse line blot assay allowed simultaneous detection of various *Babesia* and *Theileria* species at the same time using an 18S rRNA PCR followed by hybridization to species specific probes (Gubbels et al. 1999). A *T. parva* specific PCR of the p104 gene followed and was also based on a p104 specific probe (Skilton et al. 2002). This assay was recently modified to a nested-PCR assay based on p104 (Odongo et al. 2010).Loop-mediated isothermal amplification (LAMP) assays for detection

of *Theileria parva* that targets the PIM and p150 genes were also developed (Thekisoe et al. 2010).

The twenty-first century saw a technology revolution of automation and motorization of previously human controlled processes such as DNA extraction and real-time PCR analysis. These developments epitomized modern diagnosis of the carrier state of *T. parva* infected buffalo.The first assay using these new technologies were a real-time hydrolysis probe PCR assay developed at Onderstepoort Veterinary Institute (Papli et al. 2011). This assay used a universal *Theileria* primers and a *T. parva* specific hydrolysis probe. Currently in South Africa the diagnosis of carrier animals consists of a real-time hybridization PCR in combination with the IFAT. The real-time PCR was initially developed with *Theileria* genus specific primers that amplified a 230bp fragment of the 18S rRNA gene (Sibeko et al. 2008). Sensitivity of the test was compromised in the case of mixed infections with other *Theileria* species, which subsequently led to the design of a *T. parva* 'specific' forward primer. The combination of the *T. parva* 'specific' forward primer and *Theileria* genus reverse primer amplified a shorter 166bp fragment but also two other *Theileria* species: *T.*sp.(buffalo) and *T.*sp. (bougasvlei), hereafter collectively referred to as *T.* sp. (buffalo)-like parasites, because of their sequence similarity to *T. parva* (Sibeko et al. 2008; Zweygarth et al. 2009; Mans et al. 2011a& b; Pienaar et al. 2011a& b).The use of two sets of hybridization probes was incorporated to enable the discrimination between *T. parva* and *T.* sp.(buffalo), based on the differences obtained in the melting curve profiles (Sibeko et al. 2008). However, the presence of mixed *Theileria* infections with *T.*sp.(buffalo) makes interpretation of results problematic because of aberrant amplification and melting peak profiles and can lead to the misdiagnosis of *T. parva* carrier animals as being false positive or false negative (Sibeko et al. 2008; Pienaar et al. 2011a; PVVD diagnostic archival material). However, to date no systematic investigation has been conducted to quantify and understand the effect and causes of mixed-infections on the current real-time PCR assay for *T. parva.*

1.6 Research Hypothesis

The sensitivity and specificity of the current real-time hybridization probe PCR is affected by mixed infections with *T.* sp. (buffalo) and *T.* sp. (bougasvlei) to the extent that carrier animals might be misdiagnosed.

1.7 **Research Aims**:

1.7.1To determine the extent of mixed-infections within an endemic area of *T. parva* and *T.* sp. (buffalo)-like parasites.

1.7.2. To develop assays for *T. parva* that is independent of the real-time hybridization assay to determine whether mixed-infections affect the real-time assay.

1.7.3. To determine which *T.* sp. (buffalo)-like genotype is the major contributing factor in mixed-infection related misdiagnosis and investigate the relationship between the *T.* sp. (buffalo)-like genotypes.

1.7.4. To develop a real-time PCR assay for *T. parva* that is not affected by mixed-infections.

1.7.5. To develop real-time PCR assays to discriminate between *T.* sp. (buffalo) and *T.* sp. (bougasvlei).

1.7.6. To determine the incidence and distribution of both *T.* sp. (buffalo) and *T.* sp. (bougasvlei).

1.7.7.To confirm or reject the hypothesis that *T.* sp. (buffalo) and *T.* sp. (bougasvlei) are different species.

CHAPTER 2

Independent molecular diagnostic assays for Theileria parva

**(Part of the work presented in this chapter was accepted for publication in Parasitology. Pienaar et al. 2011a as well as in the proceedings of the 1st Annual International Conference on Advances in Veterinary Science Research 2013)*

2.1 INTRODUCTION

Theileria parva is the causative agent of buffalo derived Corridor disease, cattle derived East Coast fever (ECF) and Zimbabwe theileriosis (Neitz, 1955; Norval et al. 1992). In African buffalo (*Syncerus caffer*) the parasite typically does not cause disease, however; they remain carriers with low parasitic levels once infected (Norval et al. 1992). A carrier state may be life-long with the longest documented carrier period in a vector free area being twenty years (F. T. Potgieter, Personal communication, 2010). Two of the three known vector ticks for *T. parva*, *Rhipicephalus appendiculatus* and *R. zambeziensis* occur sympatrically in South Africa. With Corridor disease being a controlled disease in South Africa, preventative measures are taken to prevent the establishment of a carrier state of buffalo-derived *T. parva* in recovered cattle under field conditions (Potgieter et al. 1988; Laubscher et al. 2012). Therefore, buffalo has to be certified 'disease free' for translocation purposes between and within endemic and non-endemic regions in South Africa.

Currently the diagnosis of infected buffalo consists of a real-time hybridization PCR in combination with the indirect immune-fluorescent antibody test (IFAT). The diagnosis of carrier buffalo evolved from using the indirect immune-fluorescent antibody test (IFAT) to various molecular based tests involving the amplification of the18S rRNA gene by means of a set of universal *Theileria* genus primers and a slot blotting hybridization technique, to real-time polymerase chain reactions (PCRs) (Allsopp et al. 1993; Collins et al. 2002; Sibeko et al. 2008 Papli et al. 2011; Pienaar et al. 2011a). The specificity of the current real-time hybridization PCR is compromised in that it amplifies two other *Theileria* species as well: *T.* sp.(buffalo) and *T.* sp. (bougasvlei), hereafter collectively referred to as *T.* sp. (buffalo)-like parasites (Sibeko et al. 2008; Zweygarth et al. 2009; Pienaar et al. 2011b).To enable discrimination between *T. parva* and *T.* sp. (buffalo)-like organisms, two sets of hybridization probes were incorporated (Sibeko et al. 2008). However, the presence of mixed infections with *T.* sp.(buffalo)-like parasites made interpretation of results problematic due to aberrant amplification and melting peak profiles, that increased the probability of misdiagnosing *T. parva* carrier animals (Sibeko et al. 2008; Pienaar et al. 2011b). Suppression of PCR signal with a subsequent interpretation of samples as false-negative has also been indicated (Pienaar et al. 2011b). Markers independent of the 18S rRNA gene could resolve

problematic results (Pienaar et al. 2011a), and protein markers were identified that included, the p67 piroplasm surface protein, protein genes from the *Tpr* multi-copy locus and p104 (Baylis et al. 1991, Bishop et al. 1997, Iams et al. 1990, Nene et al. 1996).

Two of the protein markers (p67 and p104) occur as a single gene on chromosome three and four respectively, are syntenic in the *T. parva* and *T. annulata* genomes, and therefore considered to be orthologs (Gardner et al. 2005). *Tpr* on the other hand, exist as a multi-gene family of thirty three gene members in the genome of *T. parva*(Painet al.2005). It has more than twenty three copies arranged in tandem on chromosome three with a high level of sequence identity in the C-terminal region, thought to be maintained by concerted evolution (Bishop et al. 1997). The *T. annulata* related gene (*Tar*) has seventy two gene members dispersed through the genome on all four chromosomes. The *Tpr* family in *T. parva* is paralogous to *Tar* in *T. annulata* and due to its high copy number might be an attractive target for diagnostic use with regard to sensitivity. As such, being able to harness these markers either as conventional PCR methods or high throughput qualitative methods will aid in the unambiguous diagnosis of carrier buffalo, particularly in the case of mixed infections (Pienaar et al. 2011a). In this regard, the use of real-time PCR has advantages over conventional approaches, specifically to prevent cross-contamination, in terms of speed and reproducibility within a routine diagnostic laboratory.

Whilst the 18S rRNA based real-time PCR is the current gold standard diagnostic test in South Africa for *T. parva* DNA detection in buffalo, its ambiguous ability to detect *T. parva* in buffalo that exhibits mixed *Theileria* infections with *T.* sp. (buffalo)-like parasites, remains problematic. The current chapter willinvestigate this by developing alternative (conventional as well as real-time SYBG Green) protein gene based assays. We demonstrate that these assays are comparable with the conventional PCR and the real-time hybridization assay to detect field carrier-animals and detect false-negative samples due to mixed-infection suppression. These assays can be employed as supplementary assays in the unambiguous diagnosis of *T. parva* infections in buffalo as they should be unaffected by the occurrence of *T.* sp. (buffalo)-like parasites along with *T. parva* parasites within the same buffalo host.

2.2 MATERIALS AND METHODS

2.2.1 Sample collection and DNA extraction

Two hundred and forty buffalo from twelve geographically separated herds in the KNP were sampled (Fig.2.1). These herds are representative of the endemic area of the parasite and its vector ticks. The QIAmp DNA mini kit (QIAGEN, Hilden, Germany) was used for the extraction of genomic DNA from 200 microliter (µl) of whole blood according to the manufacturer's instructions. DNA was eluted in 100µl of the low-salt buffer provided in the kit as recommended for long term storage. A 10 fold serial dilution of blood from the *T. parva* gold standard positive buffalo, KNP102 (Sibeko et al. 2008), was prepared using negative bovine blood for sensitivity determination. DNA was extracted from this dilution range using the MagNa Pure (Roche Diagnostics) and the Large Volume DNA Isolation Kit (Roche Diagnostics, Mannheim, Germany). These samples were reserved for the use in determining sensitivity of the real-time protein gene assays. Analytical specificity of the assays were confirmed through the testing of samples that contained different parasites as previously identified via cloning and sequencing of the 18S gene (Mans et al. 2011a). These included: *Babesia bovis*, *Babesia bigemina*, *Trypanosoma vivax*, *T. congolense* Savannah,*T. congolense* Kilifi,*Theileria annulata*, *T.* sp. (duiker), *T.* sp. (kudu), *T.* sp. (sable), *T. buffeli*, *T. mutans*, *T. velifera* and *T.* sp. (buffalo).

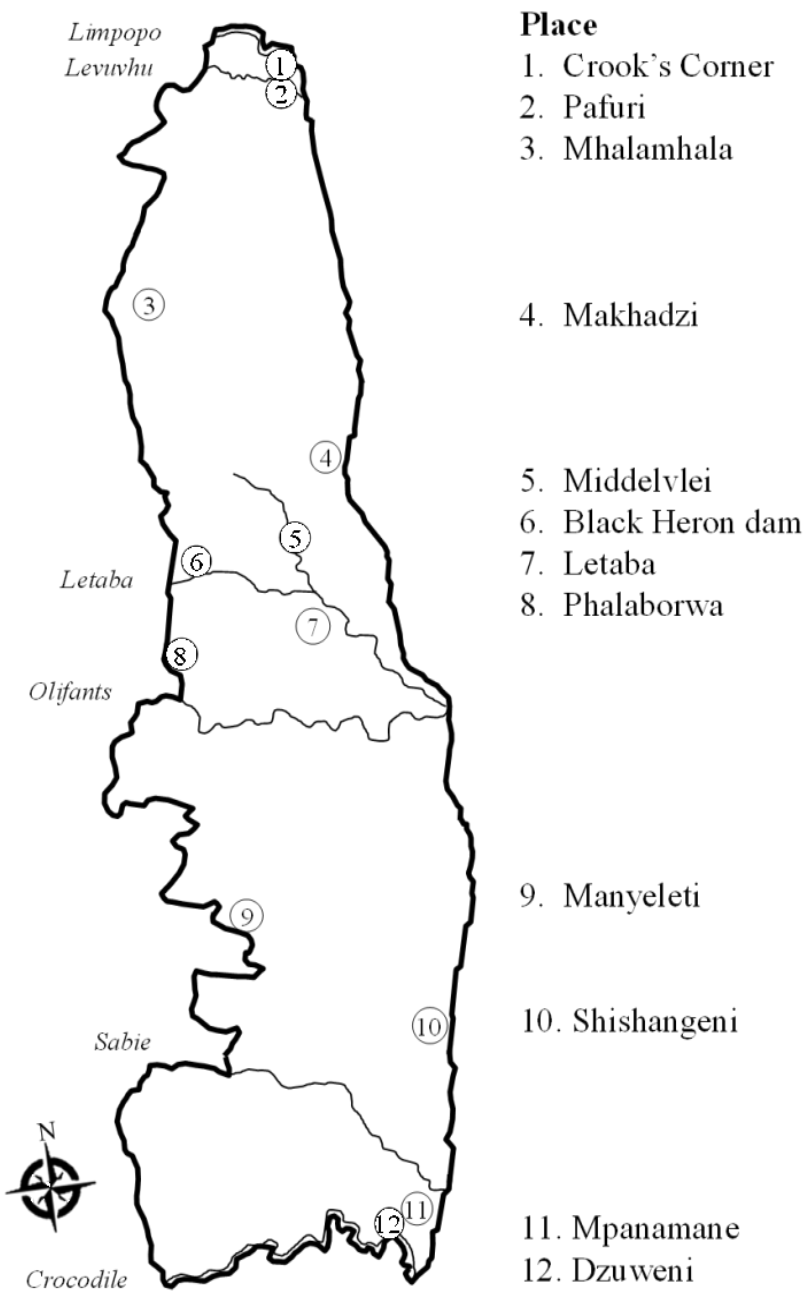


Figure 2.1: Map of the Kruger National Park showing sampled localities.

2.2.2 Real-Time hybridization screening of T. parva

The *T. parva* incidence was determined using the real-time PCR as described by Sibeko et al. (2008). Briefly, reaction conditions included 4 µl of the LightCycler-FastStart DNA MasterPlus Hybridization mix (Roche Diagnostics, Mannheim, Germany), 1U uracil deoxy-glycosylase (UDG) (Roche Diagnostics, Mannheim, Germany), 0.5 pmol forward (TpF: CTGCATCGCTGTGTCCCTT) and reverse (TgR: ACCAACAAAATAGAACCAAAGTC) primers, 0.1 pmol of the *T. parva* specific anchor and probe (*T. parva* anchor: GGGTCTCTGCATGTGGCTTAT–FL; *T. parva* sensor: LCRed640-TCGGACGGAGTTCGCT—PH), 0.1 pmol of the *Theileria* genus specific hybridization anchor and probe (*Theileria* genus anchor: AGAAAATTAGAGTGCTCAAAGCAGGCTTT–FL; *Theileria* genus sensor: LCRed705: GCCTTGAATAGTTTAGCATGGAAT—PH) pairs at a final volume of 20 µl. Crossing-point (CP) values were calculated by the automated qualitative analysis mode of the LightCycler 2.0 software (Roche Diagnostics, Mannheim, Germany). The following conditions were programmed into the LightCycler 2.0: After an initial 10 minute activation of UDG at 40°C, a 95°C pre-incubation for 10 minutes was performed before the quantifying forty five cycles of amplification with a denaturation step at 95°C for 10 seconds, annealing at 58°C for 10 seconds and extension at 72°C for 15 seconds. Fluorescence was monitored after annealing in the 640nm channel. The amplified products were subjected to a melting curve analysis starting at 40°C to 95°C for 40 seconds during which fluorescence was continuously monitored at a rate of 0.2°C per second. Results were analyzed using the LightCycler 2.0 software.

The real-time hybridization test employs two hybridization probe sets for *T. parva* (640 nm) and the *Theileria* genus (705 nm).Results obtained were designated according to the following protocol (Sibeko et al. 2008; Pienaar et al. 2011a) (Fig. 2.2): negative samples do not show amplification or melting curves for the 640 or 705 nm channels. *T. parva* positive samples show amplification and melting curves in both 640 and 705 nm channels. In the case where weak amplification and melting curves are observed for the *T. parva* specific 640 nm probe, but significant signals are obtained for the 705 nm probe, the results are interpreted as being *T. parva* negative, but *T.* sp. (buffalo)-like positive.

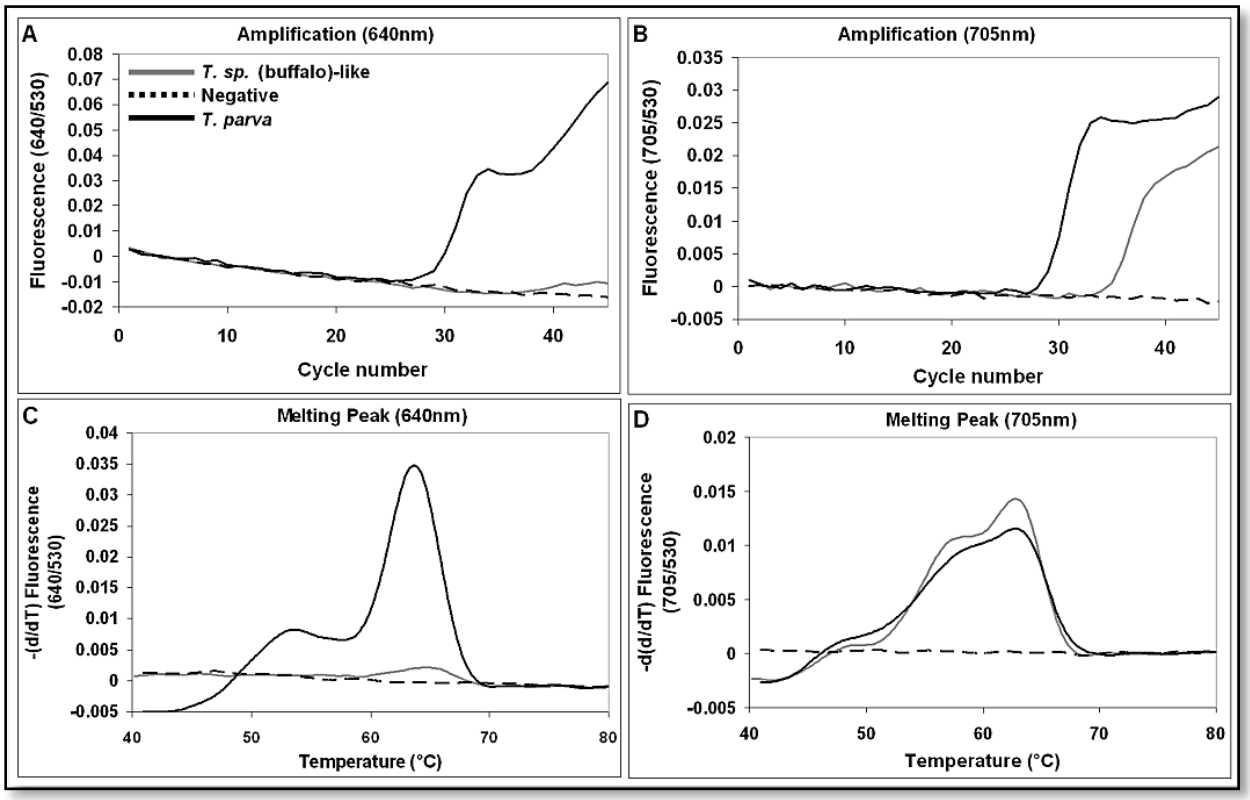


Figure 2.2:*Theileria parva*real-time PCR hybridization assay results. Indicated are the three possible result types that can be obtained, a negative (striped line), *T. parva* (black line) and *T.* sp. (buffalo)-like (light grey line) positive sample. Results are presented as amplification curves at 640nm (A) and 705nm (B), as well as melting profiles at 640nm (C) and 705nm (D).

2.2.3 Estimation of mixed infections: the reverse line blot assay

Mixed *Theileria* infections in the sample set were assessed using the reverse line blot assay as described previously (Matjila et al. 2004), with minor modifications to the PCR. The universal *Theileria* and *Babesia* primers; RLB-Forward (5'-GAG GTA GTG ACA AGA AAT AAC AAT A-3') and RLB-Reverse (biotin-5'-TCT TCG ATC CCC TAA CTT TC-3') were used to amplify a 459-522bp fragment of the V4 variable region of the 18S rRNA gene. The 50µl reaction mix consisted of 25µl GoTaq® Green Master Mix (Promega), 10pmol of each forward and reverse primer and 2.5µl DNA template.The initial 10 cycles of the touch-down PCR were modified to a 1°C decrease instead of the annotated 2°C decrease in annealing temperature. The initial 2 minute denaturation step at 94°C was followed by 10 amplification cycles with a decrease in annealing temperature of 1°C after each cycle starting at 67°C and ending at 57°C while the

denature step at 94°C (30 seconds) and extension at 72°C (30 seconds), remained the same. Hereafter followed 40 cycles of amplification with denaturation at 94°C (30 seconds), annealing at 57°C (30 seconds) and extension at 72°C for one minute. The PCR was rounded off with a final extension step at 72°C for seven minutes.Of the 50µl, 10µl of the PCR product was subjected to 2% agarose gel electrophoresis in 1xTris-acetate-EDTA (TAE) buffer for 20min at 100V and visualised under UV light. The gel was stained by adding 2µl ethidium bromide stock (10mg/ml) to 100ml agarose gel for a final concentration of 0.2ug/ml.

Sixteen *Theileria* and *Babesia* species occurring in bovines (buffalo and cattle) were selected for the synthesis of modified species specific oligonucleotide probes (Matjila et al. 2008) (Integrated DNA Technologies, Carolville, USA), (Table 2.1).These probes were diluted to 1000pmol/µl in 500uM sodium bicarbonate ($NaHCO_3$, pH8.4) and applied to the membrane after the surface carboxyl groups of the negatively charged nylon Biodyne® C membrane (Pall Gelman Laboratory, USA) were activated, rendering them susceptible for the covalent bonding of the six carbon amino-linked modified probes. This was done by covering the membrane with 16% w/v 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC or EDAC) for ten minutes.Using an ImmunoBlot blotting instrument (Hoefer® Inc, MA) each channel was filled with a respective probe and aspirated after brief incubation. Hereafter, the membrane was inactivated with 100mM sodium hydroxide, washed in a pre-heated 2xSSPE/0.1%SDS buffer (2x Saline-Sodium Phosphate-EDTA/0.1% Sodium Dodecyl Sulphate buffer) at 60°C and stored in 20mM ethylenediaminetetra-acetic acid (EDTA), pH 8 at 4°C until use.

The remaining PCR product was diluted in 330 µl $NaHCO_3$, denatured at 100°C for 10 min and directly afterwards chilled on ice. The membrane was washed in pre-heated 2x SSPE/0.1% SDS buffer before application of the denatured PCR products and re-aligned into the blotting instrument with the slots perpendicular to the applied probe lanes. Each channel was filled with the denatured PCR product while empty channels were filled with $NaHCO_3$ to prevent desiccation or possible channel cross flow. Hybridization was performed at 42°C for an hour. PCR products were aspirated and the membrane subjected to high stringency washes at 50°C in pre-heated 2x SSPE/0.5% SDS buffer. A streptavidin horseradish-peroxidase conjugate was used to detect the hybridized biotinated PCR-probe complex. The conjugated product was visualized as fluorescence upon brief incubation with equal volumes of luminol and stable peroxide

(North2South Chemiluminescent Nucleic Acid detection kit – Thermo Scientific) and exposure on CL-XPosure Film (Thermo Scientific) in a dark room.

Table 2.1: Species and genus specific oligonucleotide probe sequences used in the preparation of the reverse line blot membrane.

Oligonucleotide probe	***Sequence***
Babesia divergence	ACT RAT GTC GAG ATT GCA C
Babesia microti	GRC TTG GCA TCW TCT GGA
Babesia bigemina	CGT TTT TTC CCT TTT GTT GG
Babesia bovis	CAG GTT TCG CCT GTA TAA TTG AG
Babesia major	TCC GAC TTT GGT TGG TGT
Theileria sp.(duiker)	CAT TTT GGT TAT TGC ATT GTG G
Theileria sp. (kudu)	CTG CAT TGT TTC TTT CCT TTG
Theileria sp. (sable)	GCT GCA TTG CCT TTT CTC C
Theileria bicornis	GCG TTG TGG CTT TTT TCT G
Theileria annulata	CCT CTG GGG TCT GTG CA
Theileria buffeli	GGC TTA TTT CGG WTT GAT TTT
Theileria sp. (buffalo)	CAG ACG GAG TTT ACT TTG T
Theileria mutans	CTT GCG TCT CCG AAT GTT
Theileria parva	GGA CGG AGT TCG CTT TG
Theileria taurotragi	TCT TGG CAC GTG GCT TTT
Theileria velifera	CCT ATT CTC CTT TAC GAG T
Theileria/Babesia genus	TAA TGG TTA ATA GGA RCR GTT G

2.2.4 Protein genes: conventional PCR and SYBR Green assays

The three selected protein genes included a section coding for the N-terminal as well as the C-terminal of the surface sporozoite antigen P67 gene (Nene et al. 1996), a fragment of the p104 gene (Iams et al. 1990) and a fragment of the C-terminal trans-membrane conserved region of the Tpr1 gene (Baylis et al. 1991; Bishop et al. 1997). Primers were designed and synthesized to

amplify four different gene fragments (Inqaba Biotechnical Industries (Pty) Ltd, Pretoria, South Africa).

2.2.4.1 Conventional PCR assay

Four different gene fragments specific for *T. parva* were amplified and included a portion coding for the N-terminal end of the p67 gene, p67N (GI number: 4106803; p67NF: CTA CGG AGG AAC AAC CAT TTC CTT CTA G; p67NR: CGA TGT AGT TTC ACC TGT GGA TGT TTT TCC), a portion coding for the C-terminal end of the p67 gene, p67C (GI number: 4106803; p67CF: ACA CCA GGA CGA GGA TCA TCA GGT AC; p67CR: GGT TCC ATT AGG AGC TGA AGG TGG TTG) (Nene et al., 1996), a fragment of p104 (GI number: 71028857; p104F: CTC TCC CTG AGA CAC CTG GAA CTC; p104R: GGT TTC TTT GGC TTC GAT GGC CTC G) (Iams et al., 1990) and a fragment of the C-terminal transmembrane conserved region of the *Tpr*1 gene (GI number: 71026055; TprF: TGA CCT AGT GAT TCC CAC CAT GAT CA; TprR: AAT GCT GCT AGG TTC TTA TTG CAG TTC) (Baylis et al., 1991; Bishop et al., 1997; Gardner et al., 2005). Primer specificity was tested by BLAST analysis against other selected databases. Potential self-complementarity was determined to eliminate the possibility of self-annealing and dimer formation using OligoCalc (Kibbe, 2007). All primers were designed to have average similar properties and melting temperatures (Tm) with the aim of using the same PCR conditions for all four primer sets. Expected amplicon sizes were predicted based on the selection criteria using the multiple sequence alignment editor GeneDoc (Nicholas et al. 1997).

Conventional touch-down PCR was performed using an initial denaturation step at 94°C (2 minutes), 10 cycles of a touch-down procedure that included 30seconds denaturation at 94°C, 30seconds annealing starting at 68°C and decreasing 1°C with each cycle up to 59°C, with 30seconds extension at 72°C. This was followed by 35 cycles of denaturation at 94°C (30seconds), annealing at 59°C (30seconds) and extension at 72°C (1 minute). A final seven minute extension step at 72°C was performed to ensure that all PCR products were fully extended. Reaction conditions included the use of 25µl GreenTaq Ready reaction mix (Promega), 2.5µl of DNA template and 10pmol of each primer up to a total volume of 50µl.PCR

products were analyzed by conventional 2% agarose gel electrophoresis using standard TAE buffer and visualized using ethidium bromide.

2.2.4.2 Cloning and sequencing of amplified products

To confirm the identity of the amplified products, 1 µl product was ligated overnight at 10°C into the pGEM®-T Easy Vector (Promega) using 1 µl vector, 5 µl 10x ligation buffer and 2 µl water. The enzyme;T4 DNA Ligase, catalyzes the joining of two strands of DNA between the 5´-phosphate and the 3´-hydroxyl groups of adjacent nucleotides in either a cohesive-ended or blunt-ended configuration.For transformation, 1 µl of the ligated product were incubated with 10 µl competent *Escherichia coli* Ecloni cells (Lucigen® Corporation) on ice for 20 minutes before heat shock for 1 minute at 42°C. Cells were placed on ice for 3 minutes before addition of 100 µl recovery medium followed by incubation at 37°C with shaking at 250 rpm for 1 hour. The medium were then plated on imMedia™ Amp Blue agar plates that contain X-Gal and ampicillin (Invitrogen) and incubated overnight at 37°C. White colonies were picked and resuspended in 20 µl water before lysed at 94°C for 10 minutes. Colonies were screened using the M13 forward M13F (5'-GTA AAA CGA CGG CCA GTG AAT-3') and reverse M13R (5'-CAG GAA ACA GCT ATG ACC ATG-3') vector primers using a conventional PCR. The PCR reaction were composed of 25 µl GoTaq® Green Master Mix (Promega), 10 pmol of each primer, 2.5 µl DNA template made up to a final 50 µl with PCR grade water. PCR conditions included an initial two minute denaturation step at 94°C followed by denaturation at 94°C (30 seconds), annealing at 51°C (30 seconds) and extension at 72°C (one minute) with a final extension step of 7 minutes at 72°C. Amplified products were analyzed by agarose gel electrophoresis as described in section 2.2.3. Amplicons from positive samples were purified using the Silica Bead DNA Gel Extraction Kit (Thermo Scientific) following the manufacturer's instructions.

Purified products were sequenced from either end using the M13F or M13R primers, respectively using the ABI PRISM® BigDye™ Terminator v3.0 Ready Reaction Cycle Sequencing Kit. Sequencing reactions were composed of 1 µl 3.2 pmol/µl primer, 1 µl ready reaction mixture, 4 µl 5x buffer, 13 µl water and 1 µl purified template. The sequencing PCR consisted of 25 amplification cycles of denaturation at 96°C (10 seconds), annealing at 51°C (5 seconds) and extension at 60°C (4 minutes) and storing at 4°C. Sequencing reactions were

precipitated by adding 2 µl 3M sodium acetate pH 5.2, 2 µl 125 mM EDTA, 50 µl absolute ethanol, mixing and centrifuging for 20 minutes at 14000xg in a microcentrifuge at room temperature. Supernatant were removed and precipitates washed with 200 µl 70% ethanol and pelleted for 10 minutes at 14000xg in a microcentrifuge at room temperature. Supernatant were removed and the pellet dried for 10 minutes at 94°C. The reaction was then submitted to the Onderstepoort Veterinary Institute Sequencing Facility for lane service on their ABI3100 sequencer. Sequences were analyzed by BLASTN analysis (Altschul et al. 1990).

2.2.4.3 SYBR Green PCR assay

A 20 µl reaction mix was pipetted into a 96 well reaction plate consisting of 0.5 pmol of the forward and reverse primer, 10 µl of the 2x hot start SYBR Green master mix (LightCycler® 480 SYBR Green I Master kit, Roche Diagnostics, Mannheim, Germany), and 1U of UDG and sealed with a colorless adhesive strip. The same touch-down conditions were programmed into the LightCycler® 480 as described for the conventional touch-down PCR. Results were analyzed using the LightCycler software Version 1.5.0. Amplification consisted of an initial two minute denaturation step at 94°C for two minutes followed by a touch-down PCR with 10 amplification cycles of denaturation at 94°C (30 seconds), annealing starting at 68°C with a decrease of 1°C to 57°C after each cycle, and extension at 72°C (1 minute). Hereafter 35 amplification cycles followed and consisted of 94°C (30seconds), 59°C (30 seconds) and 72°C (one minute) with a final extension step of 7 minutes at 72°C.

2.2.4.4 Specificity and sensitivity of the SYBRGreen assay

The KNP102 10-fold serial dilutions were used to determine sensitivity and specificity of each assay was determined using the samples identified in section 1 under Materials and Methods.

2.3 RESULTS

2.3.1 Real-time hybridization

The *T. parva* forward and *Theileria* reverse primer set amplified the expected 166 bp fragment. Analysis by agarose gel electrophoresis also indicated the amplification of several non-specific bands in different control samples (Fig. 2.3).

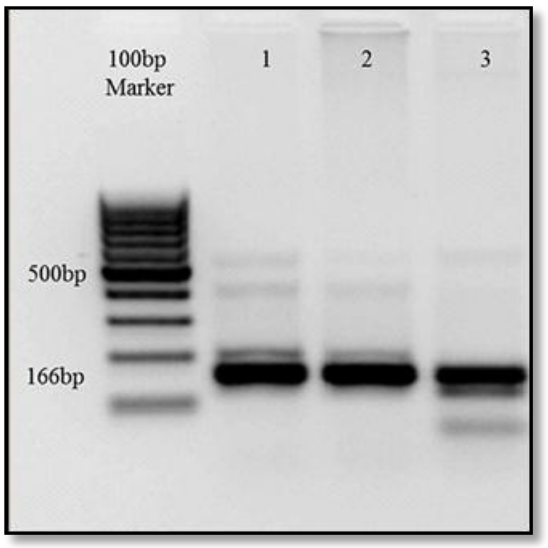


Figure 2.3: Ethidium bromide stained agarose gel indicating the real-time hybridization PCR 166 bp amplified fragment. Lanes one to three represent *T. parva* positive control samples compared against a 100 base pair DNA Ladder.

The sensitivity of the real-time hybridization test was determined using the 10x dilution range of the gold standard positive control KNP102 and was found comparable to its previously determined parasitemia of 0.003-0.001% (Sibeko et al. 2008, Papli et al. 2011). Amplicon fluorescence was detected up to a thousand fold dilution. Melting curve profiles for all dilutions showed a specific melting peak at ~63°C and a shoulder peak at ~51°C based on fluorescence detected in the 640 nm channel. The 705 nm channel showed a broad peak with optimum at 64°C (Fig. 2.4).

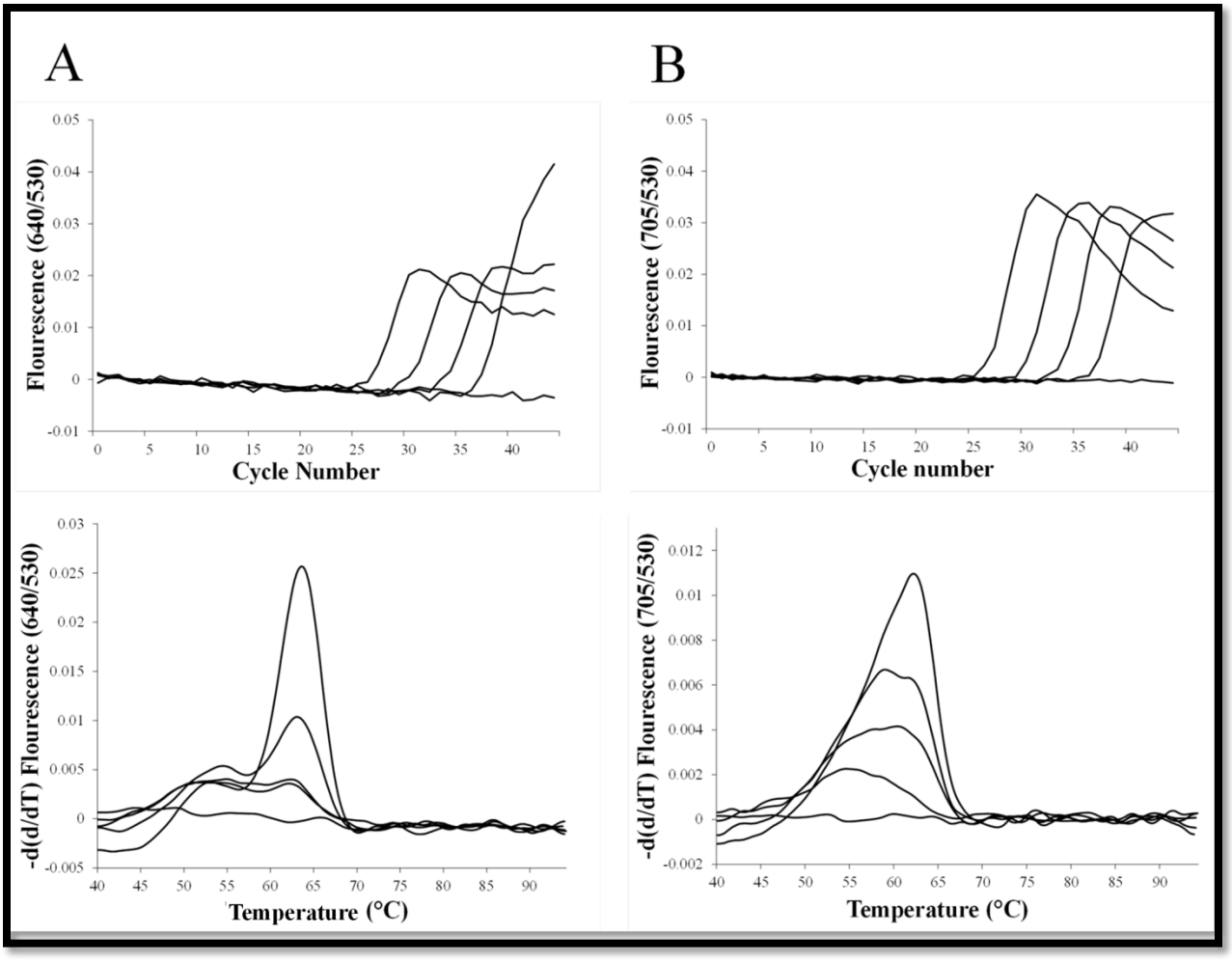


Figure 2.4: Indicated are the amplification profiles and extrapolated melting peaks of the 10x serial dilution of the *T. parva* positive gold standard KNP102. Fluorescence was detected to the third dilution in the 640 nm (A) and 705 nm (B) detection channels.

The results generated after testing the buffalo sample set were grouped in three categories based on fluorescence detected from either the 640 nm *T. parva* probe or the 705 nm *Theileria* genus probe along with the melting curve profiles as was explained in section 2.2.2. Using these criteria, approximately 16% of the animals tested negative, 72% were *T. parva* positive while 12% were *T.* sp. (buffalo) positive (Table 2.2).

Table 2.2: Real-time hybridization probe test results.

Real-time Hybridization test (n=240)		
Negative (640nm/705nm)	40/240	16%
T. parva positive (640nm/705nm positive)	173/240	72%
T. sp. (buffalo) (640nm negative/705nm positive)	28/240	12%

2.3.2 Reverse line blot

Primers targeting the conserved regions of the 18S rRNA genes for *Theileria* and *Babesia* species were used to amplify a 460 to 520 bp fragment of the hyper-variable V4 regions. In some cases light bands with smears were obtained, however most of these samples did present results (personal observation). Hybridization of a sample perpendicular to the species specific probes per lane enabled the simultaneous detection of different *Theileria/Babesia* parasite species per animal (Fig. 2.5). This permitted some resolution to the extent of mixed infections in these buffalo.

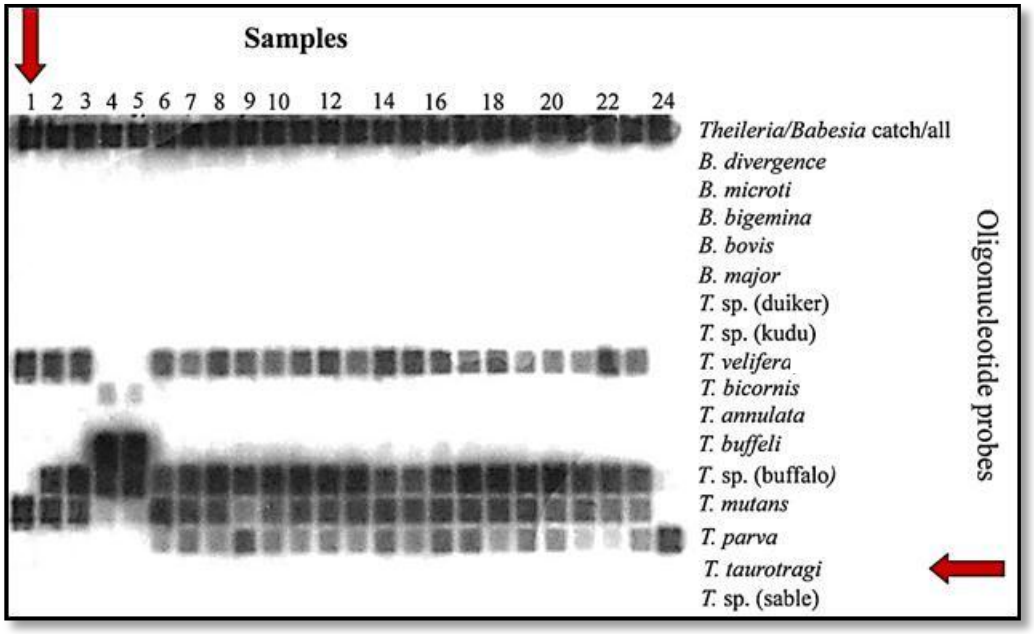


Figure 2.5: Example of a typical reverse line blot result.

Of the 240 buffalo, 210 tested positive for *T. mutans* (88%), 66 for *T. parva* (27%), 165 for *T. velifera* (81%), 105 for *T.* sp. (buffalo) (44%), 1 for *Babesia bovis* and 9 animals showed *Theileria*/*Babesia* catch/all positive results only (Fig. 2.6). One hundred and seventy three (72%) animals tested negative for *T. parva* on this assay. In some instances up to four species per animal were detected.

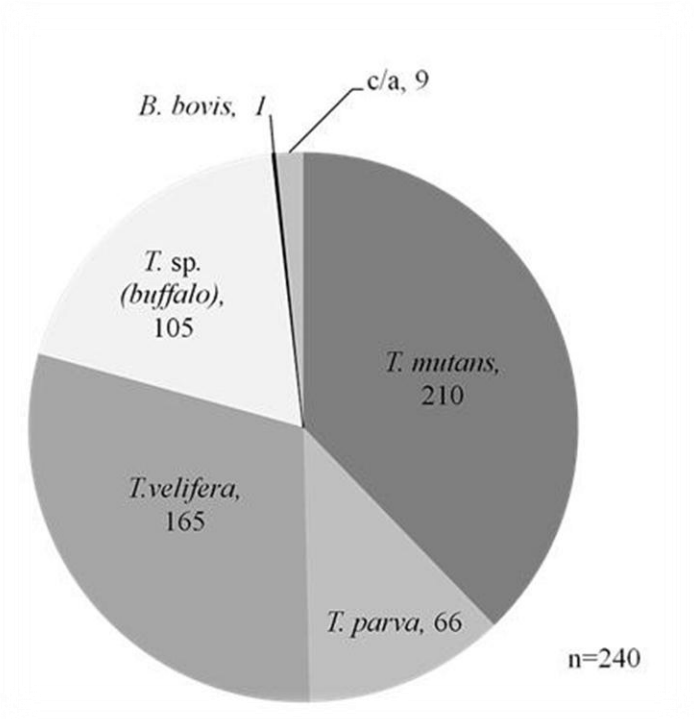


Figure 2.6: A pie chart comparing the portion of the different *Theileria* parasites found in each sample to the total of 240 animals.

2.3.3 Protein gene PCR assays

BLASTN analysis of the sequences obtained after cloning and sequencing of the conventional PCR derived amplicons confirmed the specificity of each primer set. The primers amplified ~600 bp, 300 bp, 634 bp and 393 bp for p67N and C-terminals, p104 and the *Tpr*1gene, respectively (Fig 2.7) and were specific for *T. parva*. No amplification was observed with other *Theileria* species found in buffalo: *T. mutans*, *T. buffeli*, *T. velifera*, *T.* sp. (sable), *T.* sp. (buffalo) or *T.* sp. (bougasvlei).

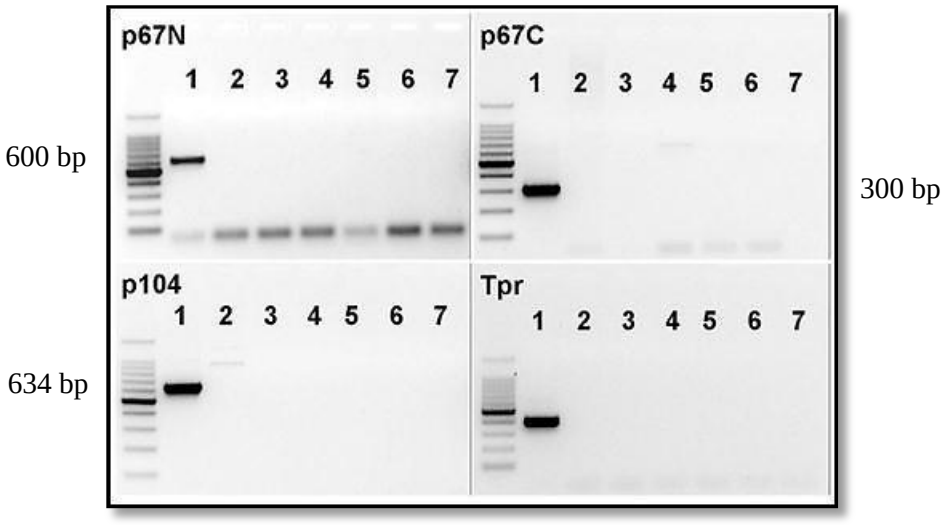


Figure 2.7: Conventional PCR analysis for p67N, p67C, p104 and *Tpr* protein genes. Lanes 1-7 indicates results for *T. parva*, *T.* sp. (buffalo), *T.* sp. (bougasvlei), *T. mutans*, *T. velifera*, *T. buffeli* and *T. taurotragi*, respectively. Molecular mass markers (100bp ladder) are included with the 500bp marker showing the highest intensity.

2.3.3.1 Sensitivity of the protein gene SYBR Green Assays

Converting the conventional PCR to the SYBR Green assay, the sensitivity of each protein gene was determined using the 10 fold serial dilution of the KNP102 positive control to generate cut-off CP values and melting curve temperatures with the use of the LC480 software (Fig. 2.8). CP values were generated by the automated software method and represent the point where the fluorescence detected in a sample curve sharply upward, also referred to as the second derivative maximum method. These values and the melting points were plotted against each other to obtain a graphical indication of the cut-off values (Fig. 2.9). The shaded areas indicate the limits for each gene specifically determined by its CP value (one cycle below the last dilution point that followed the melting peak trend) and melting curve temperature (mean Tm ± 2 degrees) (Fig. 2.9)

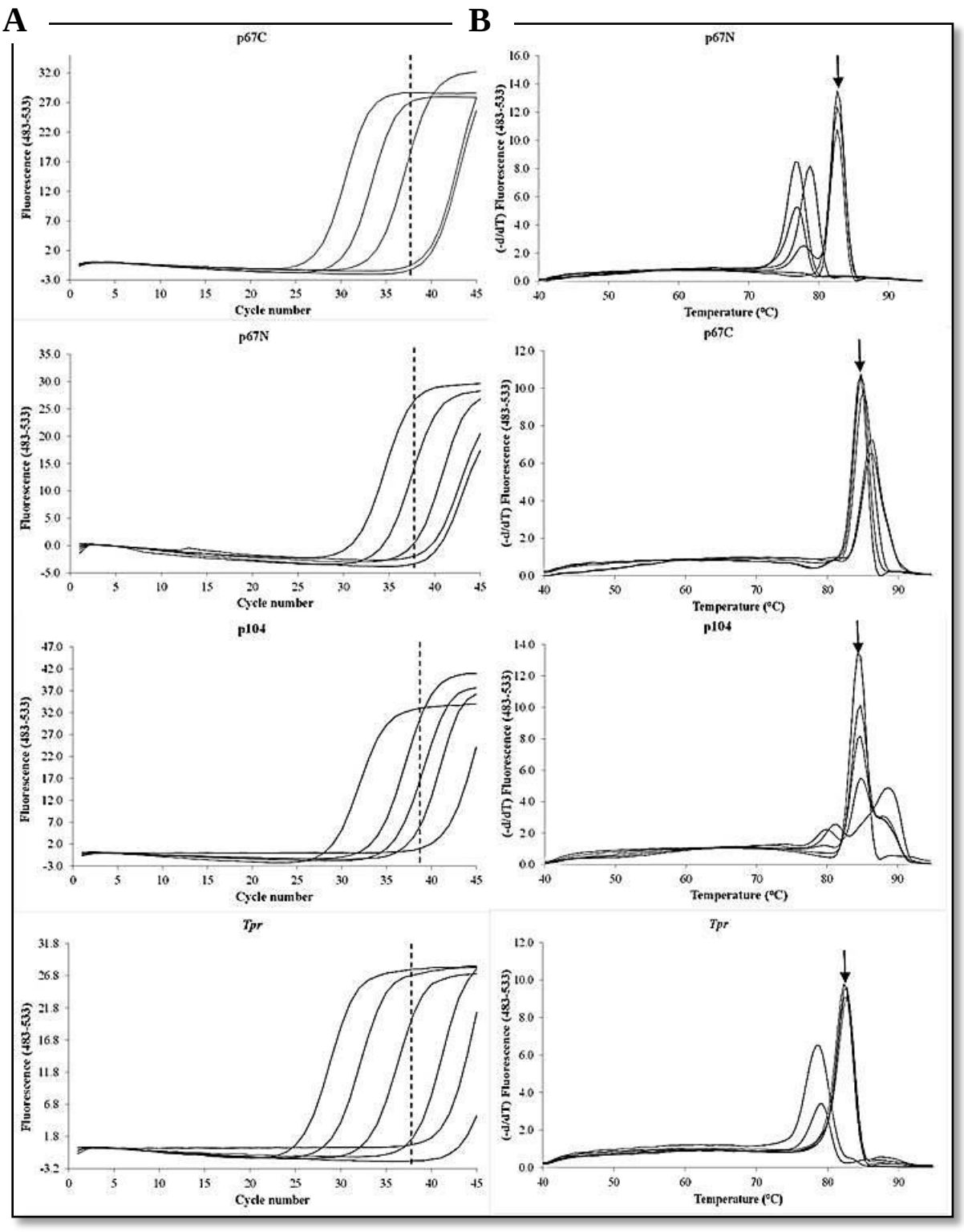


Figure 2.8: Sensitivity of the SYBR Green protein-based assays. A) Amplification curves for the SYBR Green assays. The determined cut-off point is indicated by the dashed line for each assay. B) Melting curves for the SYBR Green assays. Melting curves deviating from the cut-off point indicated by the arrow is considered negative.

The sensitivity of each protein gene using DNA from the 10x dilution range of the gold standard positive control KNP102 showed that *Tpr* and p104 were the most sensitive with positive amplifications detected up to a 1000 fold dilution corresponding to a parasitaemia of $2x10^{-6}$%. The p67C primer set amplified amplicon to a 100-fold dilution, while p67N amplified amplicon only to 10-fold dilution (Fig. 2.8). The presence of primer-dimers in the p67N PCR was significant at the higher dilution ranges. A complementarity check indicated that it had 5 possible base pairs that could allow for primer self-dimerization and possible hairpin formation. The p67N reverse primer showed no self-dimerization or hairpin formation possibilities. This validates that all genes have similar melting temperatures of 83°C-86°C within a range of generated CP values. Using the data from the detection limit, melting temperature and specificity, a cut-off range was determined for each assay that is described by a boxed area delimited by CP value (one cycle below the detection limit) and melting temperature (mean Tm ± two degrees) with p104 and *Tpr* being the most sensitive, able to amplify template up to a 1000 fold dilution (Fig. 2.8 & Fig. 2.9).

2.3.3.2 Specificity of the protein gene SYBR Green Assays

To assess the specificity of each assay, a sample set identified by the RLB, confirmed through cloning and sequencing analysis, which included *Babesia bigemina* and *B. bovis*, *T. buffeli*-like C, *T. buffeli* type D-like, *T. mutans*, *T. mutans* like-1, *T. mutans* like-2, *T.* mutans like-3, *T. mutans* MSD, *T.* sp. (bougasvlei), *T.* sp. (sable), *T. taurotragi*, *T. velifera*, *T. velifera*-like A and *T. velifera*-like B were tested using each assay (Fig. 2.9). All gave CP values and melting points that fell outside the determined cut-off area confirming that these assays were specific for *T. parva.*

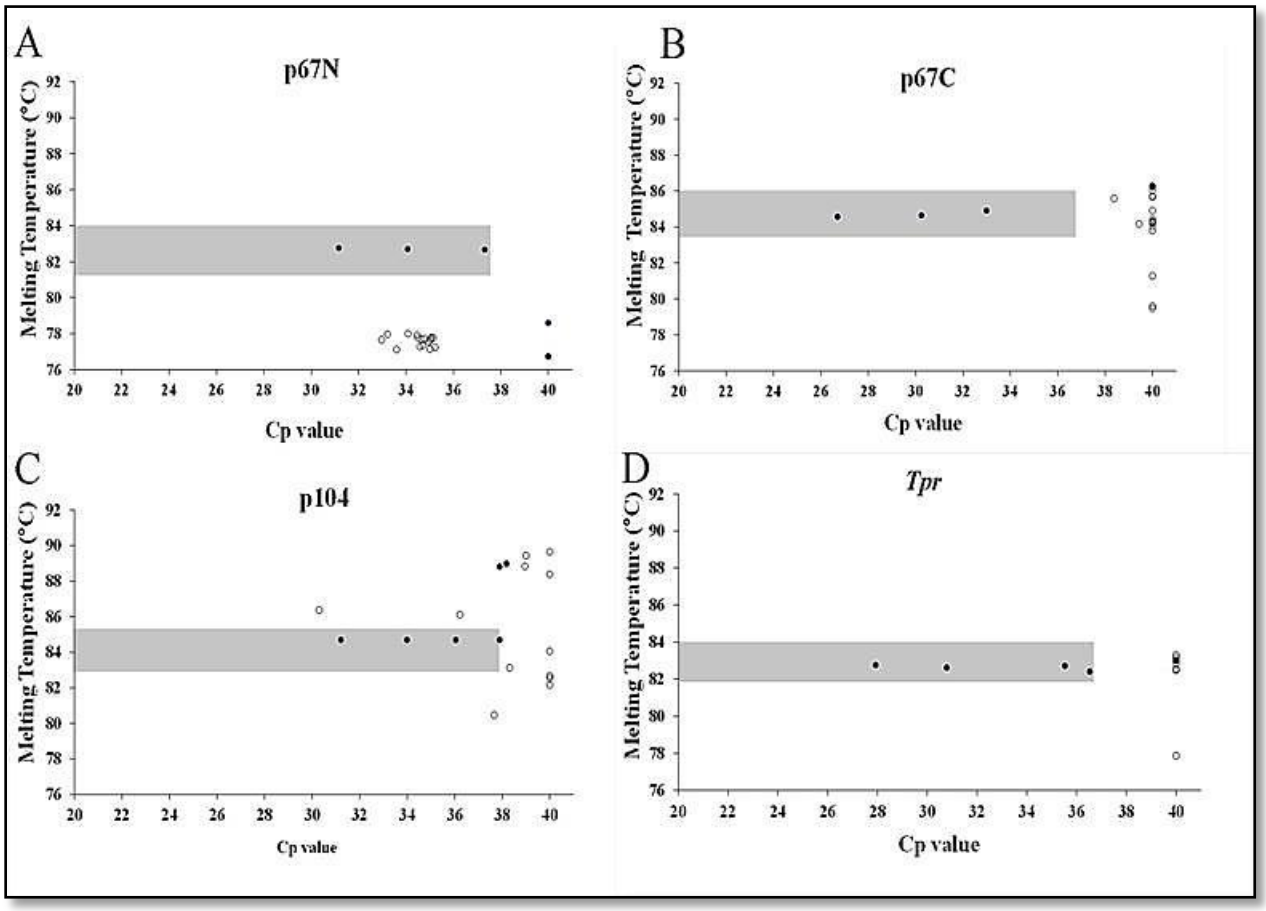


Figure 2.9: Specificity for the SYBR Green assays. The CP values plotted against the melting curve temperature values obtained for each protein gene using the KNP102 serial dilution range is indicated by black dots for A: P67N, B: P67C, C: P104 and D: *Tpr*. Shaded regions indicate cut-off criteria to designate a sample as *T. parva* positive. The white dots represent non-specific PCR product.

2.4 Analysis of field samples

Analysis of the KNP buffalo sample set using the SYBR Green analysis, indicated consistent logarithmic amplifications with melting curves observed at the expected temperatures of 83°C-85°C. However, a number of amplified field samples had CP values below the pre-determined cut-off and melting temperature profiles outside the pre-determined range (Fig. 2.10). The cut-off area therefore serves as strict criteria for the detection of *T. parva* DNA.

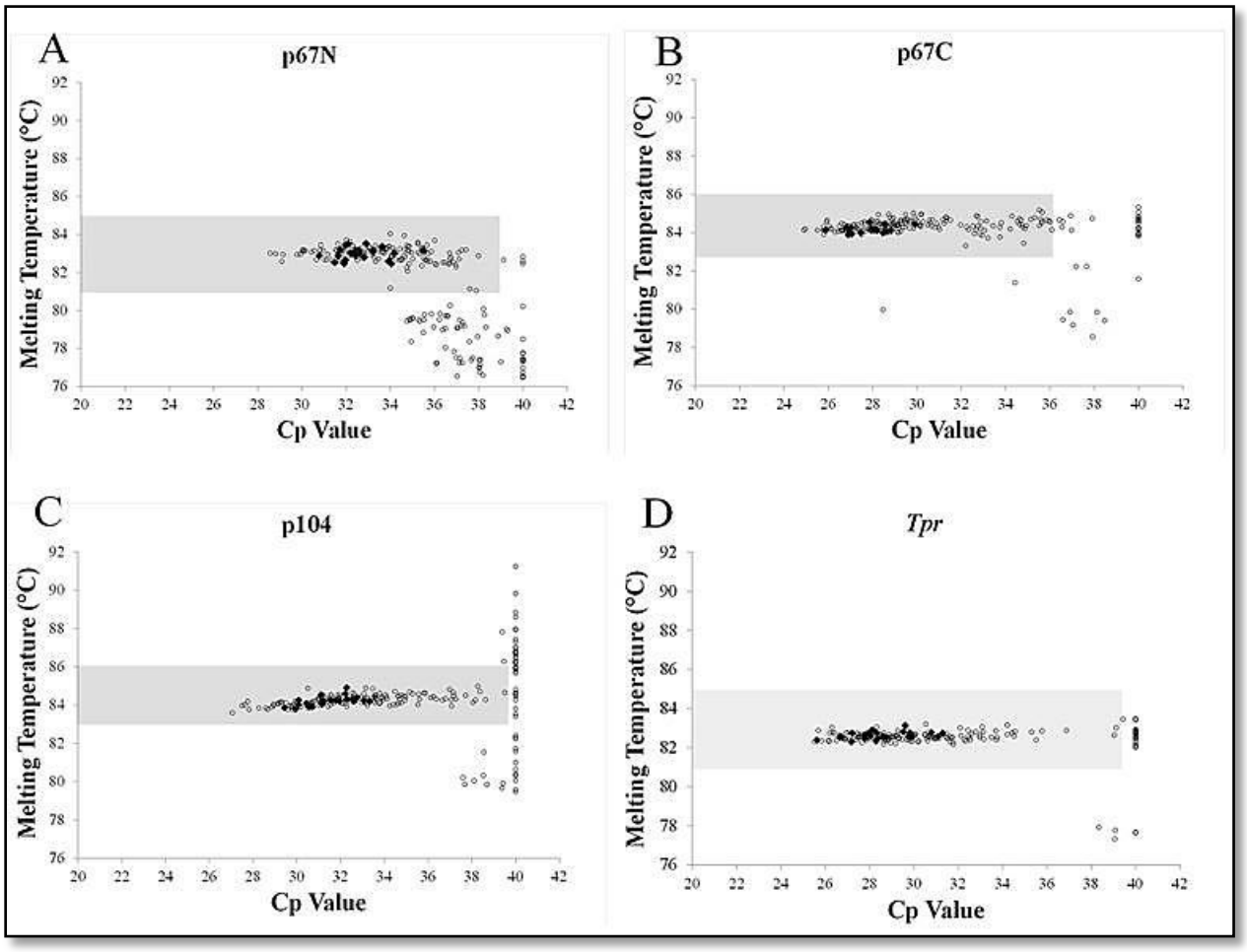


Figure 2.10: Testing of field samples using the SYBR Green assays. Shaded regions indicate samples designated as *T. parva* positive based on cut-off criteria. Black diamonds represent samples that tested *T.* sp. (buffalo)-like positive but *T. parva* negative using the hybridization assay.

The SYBR Green results were compared to previous results obtained with the RLB, hybridization and conventional PCR assays, with the hybridization assay being considered the gold standard. All assays detected 100% of samples (n=40) as being *T. parva* negative and this included 12% samples (n=28) that tested *T.* sp. (buffalo)-like positive with the hybridization assay (Fig. 2.11). The RLB assay only detected 27% *T. parva* positive compared to the 72% detected by the hybridization assay. In contrast, the SYBR Green protein PCR assays detected for p67N: 69%, p67C: 75%, p104: 74% and *Tpr*: 67% positive compared to the hybridization assay. Samples detected by the hybridization assayas *T.* sp. (buffalo)-like positive, but were

negative by the protein assays comprised 64-68% (n=18-19) and would be considered to be true-negatives. All of the latter tested consistently negative for all the protein assays. The correlation of positive samples between the different protein based assays ranged from 80-95%. However, correlation between p67N, p67C and p104 ranged from 91-97%, indicating that the *Tpr* gene detected fewer positive samples. None of the protein samples tested positive for samples negative on the Hybrid II assay (Discussed in Chapter 3).

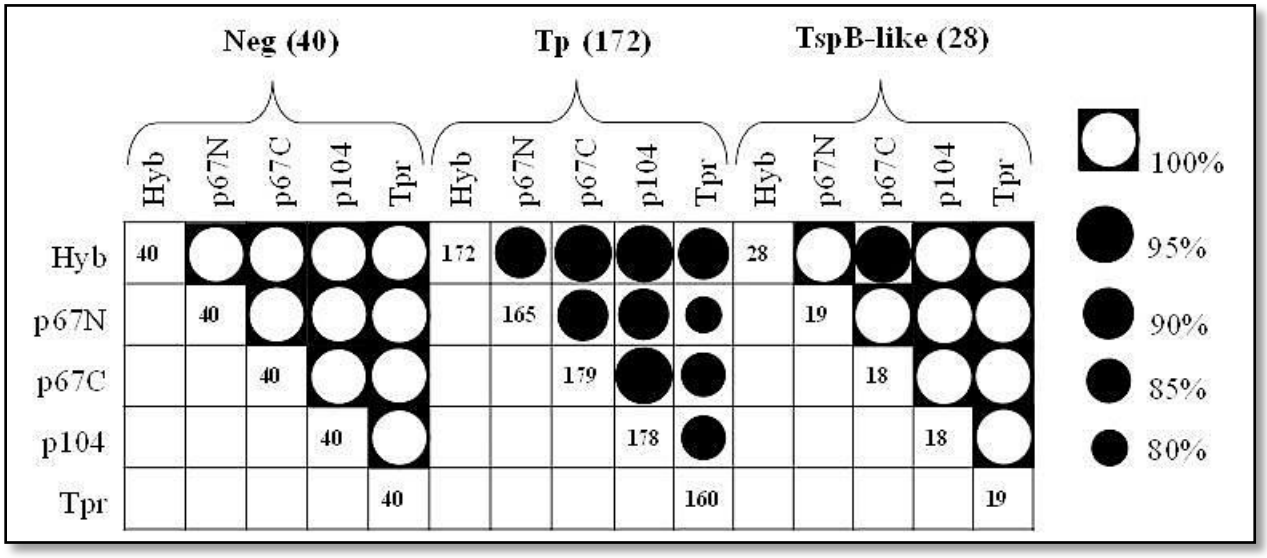


Figure 2.11: Correlation between different PCR tests. Indicated is the percentage correlation between various tests. Samples were grouped as those negative on the hybridization assay (Neg), positive for *T. parva* (Tp) and positive for *T. sp.* (buffalo)-like (TspB). Numbers in brackets indicate the number of samples analyzed for each test. Actual positive numbers for each test are indicated.

However, 29-32% (n=8-9) of the samples that tested negative with the hybridization assay, but *T.* sp. (buffalo)-like positive, consistently tested positive with all the protein genes, with non-ambiguous CP and melting peak values (Fig. 2.10). These same samples also tested positive using the conventional PCR assay. These samples all tested positive for *T.* sp. (buffalo) using the RLB test, indicating that they had mixed-infections.

2.5 DISCUSSION

Being able to accurately discriminate between the *Theileria* species infecting buffalo are of great importance not only because of disease control but because of long term economical and epidemiological implications. Diagnosis of *T. parva* is problematic due to the co-ocurrence of mixed *Theileria* infections harbored by carrier buffalo. This is further complicated by prolonged or lifelong carrier states with fluctuating parasitemias (Norval et al. 1992). These influencing factors are evident in aberrant results corresponding to PCR template competition with suppressed amplification and melting curve profiles (Sibeko et al. 2008; Pienaar et al. 2011a). In the current study, buffalo from the Kruger National Park was selected because herds from this Park are well established, free-ranging and continuously exposed to tick challenge. Most importantly, the Park is situated in the historically and current endemic area of *T. parva* and its vector tick's distribution. Comparative data were generated from two-hundred and forty buffalo using the hybridization, reverse line blot, conventional PCR and SYBR Green assays (Pienaar et al. 2011a) and serves as a well characterized dataset to validate the SYBR Green protein assays.

Mixed infections were confirmed using the RLB. Four major *Theileria* species were detected; *T. mutans*, *T. parva T. velifera* and *T.* sp. (buffalo). This confirmed buffalo to harbor, on average, up to four different *Theileria* parasites at a time. Of interest is the fact that the portion of mixed infections of *T. parva* with *T.* sp. (buffalo) is remarkably higher than determined with the Real-time PCR alone. With the Real-time PCR 12% of the population were infected with *T.* sp. (buffalo) whereas with the RLB *T.* sp. (buffalo) infections were 3.5 times higher (44%). The reverse was observed for *T. parva* infections; the 72% positives, as detected by the Real-Time PCR, dropped significantly to 27% on the RLB. This would suggest that a high number of the *T. parva* positive samples on the real-time PCR also harbor *T.* sp. (buffalo) infections.

The overall incidence of *T. parva* in the KNP buffalo using a variety of real-time assays seemed relatively high, compared to previous studies (Allsopp *et al.* 1999). This latter study only found ~33% of buffalo from the KNP to be *T. parva* positive. However, it should be noted that that study used a blotting approach comparable to the RLB in the current study. In most serological studies from buffalo, IFAT analysis found 100% of animals to be positive for *T.*

parva (Allsopp *et al.* 1999). The 69-75% of *T. parva* positive buffalo is therefore not un-expected, since the KNP is endemic for the tick vectors *R. appendiculatus* and *R. zambeziensis* and *T. parva*.

Good correlation of *T. parva* positive incidences was obtained with the hybridization assay and the protein gene assays even though the sensitivity of the protein-based SYBR Green assays are an order of magnitude below the hybridization assays (Sibeko et al. 2008; Pienaar et al. 2011a). This could be due to the parasitemia ranges for *T. parva* in the KNP, which was determined to range from 0.0001-0.1% (Chapter 3), which is well above the detection limits of the various protein based assays. Importantly, the protein based assays detected *T.* sp. (buffalo) positive samples considered to be *T. parva* negative using the hybridization assay. The protein based assays could therefore be important to resolve *T. parva* positive status in samples where mixed-infections occur.

Some hybridization PCR positive samples were false-negative on the protein based assays. A possible reason could be sequence variations in the primer area as alleles are known to exist (Sibeko et al. 2010). Development of a hybridization or hydrolysis probe assay based on these genes could therefore lead to an even higher false negative detection rate due to variation in the probe regions. The use of SYBR Green limits sequence variation effects to the primer regions as well as being a cheaper alternative to probe based assays. While variation in primer sequences may impede the SYBR Green protein-based assays to be implemented in routine diagnostic use, the ability of these assays to detect samples determined to be false-negatives using the hybridization assay, make them useful as supplementary assays in cases where ambiguous results are obtained using the hybridization assay. In this regard, the designed primers, specific for *T. parva*, eliminated any high background and non-specific product amplification. In order to set the strict cut off criteria for each amplified protein gene fragment using the SYBR Green real-time PCR, the CP values of the KNP102 10 fold dilution range were plotted against melting curve temperatures as generated by the Roche LC480 software. Compared against the 'gold standard' Real-time PCR test and the conventional touch-down PCR, it corresponded in sensitivity based on amplification and expected melting peaks of 82-84°C for all protein gene fragments.

Despite the fact that the real-time PCR did detect the majority of the *T. parva* positive samples, it failed in its ability to diagnose certain carrier animals which harbored mixed infections of *T. parva* and *T.* sp. (buffalo). Conversely, independent protein markers could detect the *T. parva* carrier status of these animals. As such, being able to harness these markers either as conventional PCR methods or high throughput qualitative methods will aid in the unambiguous diagnosis of carrier buffalo, particularly in the case of mixed infections. In this regard, the use of real-time PCR was superior to conventional approaches, specifically to prevent cross-contamination, in terms of speed and reproducibility within a routine diagnostic laboratory. These assays were unaffected by *T.* sp. (buffalo) coinciding within the same buffalo host.

The results from this Chapter confirmed suspicions that mixed-infections could affect the current real-time hybridization PCR assay leading to false-negative results. Possible reasons for these results will include suppression of PCR signal due to competition for PCR resources as observed for the RLB as well. The following Chapter will investigate the quantitative parameters that will determine whether PCR suppression can occur in field samples.

CHAPTER 3

*The Effect of Mixed Infections**

**(Work presented in this chapter was accepted for publication in Parasitology. Pienaar et al. 2011a)*

3.1 INTRODUCTION

Corridor disease, East Coast fever and Zimbabwetheileriosis (January disease) are related diseasesyndromes of cattle caused by *Theileria parva*(Norval et al. 1992). The brown ear ticks *Rhipicephalusappendiculatus*, *R. duttoni* and *R. zambeziensis*are considered to be the main vectors of*T. parva* with distribution ranges limited to Central,East and southern Africa (Lessard et al. 1990). In thecase of East Coast fever and January disease, sick andrecovered carrier cattle are infective to ticks, resultingin transmission of cattle-adapted *T. parva* to susceptiblecattle. Corridor disease results when buffaloadapted*T. parva* is transmitted from carrier Capebuffalo (*Syncerus caffer*) to cattle which die acutely,usually showing no carrier stages of the infection(Norval et al. 1991).East Coast fever was introduced into South Africa,presumably from East Africa, in 1902 and waseradicated by 1956 through an extensive quarantine,systematic dipping and slaughter campaign (Theiler,1904; Neitz, 1957). January disease was originallyidentified in Zimbabwe and was never consideredto be present in South Africa (Neitz, 1957). WhileCorridor disease was first identified in Zimbabwe, itsaetiology was elucidated in South Africa where it wasfirst recognized in the corridor formed by the historicHluhluwe and Umfolozi game parks (Neitz et al.1955). Bovine carrier states are recognized for EastCoast fever and January disease and were shownto occur under laboratory conditions for Corridordisease (Barnett and Brocklesby, 1966a& b; Kochet al. 1992; Neitz, 1958; Potgieter et al. 1988; Younget al. 1986). No carrier state could yet be confirmedfor bovines under field conditions in South Africa(Potgieter et al. 1988).Currently, the recognized Corridor disease endemicregions in South Africa include the KrugerNational Park (KNP), Hluhluwe-Imfolozi Park(KwaZulu-Natal) and regions between and surroundingthese areas (Potgieter et al. 1988). *Rhipicephalusappendiculatus* are, however, widespread acrossthe Northwest and Northern Provinces, as well asMpumalanga, KwaZulu-Natal and the eastern partsof Eastern Cape (Estrada-Peña, 2003). The potentialgeographical range of *T. parva* can thus be much wider if infected vectors or buffalo are introducedinto non-endemic disease regions. The expansion ofthe eco-tourism trade has made ‘disease-free’ buffaloa lucrative commodity with expansion of herdsoutside the endemic regions (Collins et al. 2002).This includes movement of ‘disease-free’ buffalofrom endemic to non-endemic regions. Such buffaloneed to be certified free of Brucellosis, Corridordisease, foot and mouth disease and tuberculosisby State Veterinarian authorities before relocationis allowed

(Collins et al. 2002). As such, Corridordisease is a controlled disease and the movementof buffalo inside and outside the endemic regions isstrictly regulated by the Department of Agriculture,Forestry and Fisheries (Animal Disease Act 1984,Act No. 35). This is mainly to prevent diseaseoutbreaks among cattle and the potential establishmentof a carrier state in cattle that will lead to asituation that resembles that of the original EastCoast fever epidemic (Yusufmia et al. 2010).With regard to Corridor disease testing, buffaloeswere initially tested using the indirect fluorescenceantibody test (IFAT) (Potgieter et al. 1988). This wasexpanded to include molecular testing using slot-blothybridization technology based on amplification ofthe 18S RNA using a universal *Theileria* genusspecific primer set (Allsopp et al. 1993; Collins et al.2002). The latter test was subsequently replaced bya real-time hybridization PCR test that amplifiesa 167 bp fragment from the V4 variable region of the18S rRNA gene using a *T. parva*-'specific' forwardand a *Theileria* genus-specific reverse primer (Sibekoet al. 2008). During the development of this test,a universal *Theileria* genus-specific primer set wasinitially used, but due to competitive PCR in mixedinfections this set was replaced with the *T. parva*-'specific' primer set (Sibeko et al. 2008). However,this primer set also amplifies the related *Theileria*spp., *T.* sp. (buffalo) and *T.* sp. (bougasvlei), designatedcollectively as '*T.* sp. (buffalo)-like' inthe current study (Sibeko et al. 2008; Zweygarthet al. 2009). As such, the possibility for competitionPCR still exists where mixed infections of *T. parva*and *T.* sp. (buffalo)-like parasites occur. We investigatedwhether mixed infections would be a relevantfactor under southern African field conditions, takinginto consideration the geographical distributions of*T.* sp. (buffalo)-like parasites and *T. parva*, as well asparasitaemia levels for the different parasites in thebuffalo host. The results show that mixed infectionsof*T. parva* and *T.* sp. (buffalo)-like parasites can havea considerable impact on accurate diagnosis ofCorridor disease status in Cape buffalo.

3.2 MATERIALS AND METHODS

3.2.1 DNA extraction and real-time hybridization assay

Cape buffalo blood samples from private game ranches as well as National Parks in South Africa (Marakele - MNP and Kruger National Park - KNP) submitted for routine *T. parva* testing

for the period 2008-2009 were used. Genomic DNA was extracted and the real-time hybridization PCR performed and analyzed as described in Chapter 2.

3.2.2 Estimation of parasitemia in database samples

To estimate parasitemia of *T. parva* and *T.*sp. (buffalo)-like parasites in buffalo blood samples, standard curves for the real-time hybridization assay were constructed. For this an 1101bp fragment of the 18S rRNA gene for *T. parva* and *T.*sp. (buffalo) were amplified, respectively, using *Theileria* genus specific primers 989 and 990 (Allsopp et al. 1993).The PCR products were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega) and quantified spectrophotometrically using a ND-1000 NanoDrop spectrophotometer. Similar DNA concentrations for *T. parva (*66 ng/ul) and *T.* sp. (buffalo) (63 ng/ul) were obtained. The DNA products were then diluted to give stock solutions of 10 ng/ul which corresponded to $1x10^{10}$ molecules/µl.

Calculations for copy numbers for various dilutions:

The molecular mass of the1101 bp amplified fragmentusing the Allsopp et al. (1993) primers was determined by using the average nucleotide base pair in DNA as 615 Daltons or g/mole (http://rh.healthsciences.purdue.edu/vc/theory/dna/index.html).

=1101 x 615 = 677 115 g/mole

Following this, the number of molecules in *x*-moles of 10ng/µl product was equal to Avogadro's number multiplied by the number of moles of the fragment:

Have 10ng/µl cleaned up product

Molecular Weight: 677115g/mole
In 10ng = $10x^{-9}$g THUS $10x^{-9}$g /677115 g/mole
= 1.48 x 10^{-14} mole
Thus the amount of molecules in 1.48 x 10^{-14} mole of 10ng product = 1.48 x 10^{-14} * $60.2x10^{23}$
= 8.89 x 10^{9} molecules
~ 9.0 x 10^{9}

But, you use 2.5µl/reaction:2.5µl * 8.89 x 10^{9}= **2.2 x10^{10} molecules/2.5µl** used per reaction.
But there are two 18S copies in the genome of *T. parva*, thus the genomic equivalent for a 2.5 µl reaction is half:
= 1.1 x 10^{10}genomic equivalents / 2.5 µl reaction
= 4.4x 10^{9} genomic equivalents/µl

Given these calculated values, dilution ranges could be determined that will span the common range used for real-time PCR (gray box below). This will include the theoretical limit and cut-off of the PCR (40 cycles = 1 molecule detected). A realistic range to use as dilution range would be from a CP value of 17-40 (dashed box within).

ng/ul	Molecules	Dilution factor	Per PCR reaction (2.5ul)	Genomic quivalents/ reaction
10	= 9.0×10^{9}	= 10X	= 2.2×10^{10}	= 1.1×10^{10}
1	= 9.0×10^{8}	= 1X	= 2.2×10^{9}	= 1.1×10^{9}
0.1	= 9.0×10^{7}	= 10^{-1}	= 2.2×10^{8}	= 1.1×10^{8}
0.01	= 9.0×10^{6}	= 10^{-2}	= 2.2×10^{7}	= 1.1×10^{7}
0.001	= 900 000	= 10^{-3}	= 2.2×10^{6}	= 1.1×10^{6}
0.0001	= 90 000	= 10^{-4}	= 225 000	= 112 500
0.00001	= 9 000	= 10^{-5}	= 22 500	= 11 250
0.000001	= 900	= 10^{-6}	= 2250	= 1125
0.0000001	= 90	= 10^{-7}	= 225	= 112.5
0.00000001	= 9	= 10^{-8}	= 22.5	= 11.25
0.000000001	= 0.9	= 10^{-9}	= 2.25	= 1.125

Dilution factor	Per PCR reaction (2.5ul)	Genomic equivalents	CP value
10^{1}	= 2.2×10^{10}	= 1.1×10^{10}	
1X	= 2.2×10^{9}	= 1.1×10^{9}	
10^{-1}	= 2.2×10^{8}	= 1.1×10^{8}	
10^{-2}	= 2.2×10^{7}	= 1.1×10^{7}	
10^{-3}	= 2.2×10^{6}	= 1.1×10^{6}	17.41
10^{-4}	= 2.2×10^{5}	= 1.1×10^{5}	21.23
10^{-5}	= 2.2×10^{4}	= 1.1×10^{4}	24.52
10^{-6}	= 2.2×10^{5}	= 1.1×10^{3}	28.18
10^{-7}	= 2.2×10^{2}	= 1.1×10^{2}	31.74
10^{-8}	= 2.2×10^{1}	= 1.1×10^{1}	35.01
10^{-9}	= 2.25	= 1.125	40.0

Due to the fact that there are two 18S rRNA copies in the genome of *T. parva* (Gardner et al. 2005), this yields 4.4 X 10^{9} genomic equivalents/µl. It can be assumed that *T.*sp. (buffalo)-like parasites will also have two copies of the 18S rRNA gene, as all *Theileria* thus far described, have this number (Pain et al. 2005). In the case of *T. parva*, the predominant number of

piroplasms is one piroplasm per red blood cell in the carrier-state or even in animals with parasitemia as high as 2-9% (Conrad et al. 1986). It is assumed that this would thus also be the lower limit for *T. sp.* (buffalo) organisms. It was also determined that *Theileria* in the piroplasm stages are haploid (Gauer et al. 1995). As such, a genomic equivalent (2 copies of the 18S rRNA gene) can be equated with one infected red blood cell. The mean red blood cell count for free-ranging African buffalo has been determined to be 10.0×10^{12} RBC/L (Beechler et al. 2009). Given this, the 2.5µl of DNA eluate used per assay is equivalent to 5µl whole blood, which would yield mean erythrocyte counts of 5×10^{7}RBC for buffalo. A theoretical percentage parasitemia can thus be calculated where the number of genomic equivalents added per assay is known.

Correlating copy number, crossing-point and percentage parasitemia

For most *Theileria* species the number of piroplasms is limited to 1 piroplasm per red blood cell.
The mean erythrocyte count for buffaloes is 10×10^{12} RBC/L (Beechler et al. 2009).
Extract 200µl of blood and elute in 100 µl, use 2.5 µl/reaction.
10×10^{12} RBC/L * 0.0002L = 2.0×10^{9} RBC/200µl blood
= 2.0×10^{9} RBC / 100 µl = 2×10^{7} RBC/100 µl blood
= 2×10^{7} * 2.5 µl = 5×10^{7} RBCs
Thus: 1µl eluate = 200 µl / 100 µl = 2µl blood.
2.5µl eluate = 2 µl * 2.5 µl = 5µl blood.
There are thus ~5×10^{7}equivalents in 2.5 µl DNA used per reaction.
Thus the percentage of genomic equivalents/infected RBCdivided by the number of RBC per PCR reaction should yield the theoretical parasitemia per dilution factor.

Dilution Factor	**Per PCR reaction (2.5ul)**	**Genomic equivalents/ Infected RBC**	**% Parasitemia**
10X	= 2.2×10^{10}	= 1.1×10^{10}	22000
1X	= 2.2×10^{9}	= 1.1×10^{9}	2200
10^{-1}	= 2.2×10^{8}	= 1.1×10^{8}	220
10^{-2}	= 2.2×10^{7}	= 1.1×10^{7}	22
Realistic parasitemias			
10^{-3}	= 2.2×10^{6}	= 1.1×10^{6}	2.2
10^{-4}	= 2.2×10^{5}	= 1.1×10^{5}	2.2×10^{-1}
10^{-5}	= 2.2×10^{4}	= 1.1×10^{4}	2.2×10^{-2}
10^{-6}	= 2.2×10^{3}	= 1.1×10^{3}	2.2×10^{-3}
10^{-7}	= 2.2×10^{2}	= 1.1×10^{2}	2.2×10^{-4}
10^{-8}	= 2.2×10^{1}	= 1.1×10^{1}	2.2×10^{-5}
10^{-9}	= 2.2	= 1.1	2.2×10^{-6}

A 10-fold serial dilution range of the *T. parva* and *T.* sp. (buffalo) 18S gene stock solutions, that gives a range of 10 – 1 X 10^7 molecules, spans the known observed CP values (15-35 cycles) as obtained by real-time hybridization assay of *T. parva* diagnostic samples (personal observation). This corresponds to calculated parasitemia percentage values of $1X10^{-5}$% -10% (Fig. 3.1). Using the equations obtained from the curve fits, estimated parasitemia can be calculated for all samples that have been previously assayed. In all real-time PCR assays, buffalo KNP102, a known *T. parva* positive infected buffalo used as gold standard positive control (Sibeko et al. 2008), was used as external control to estimate the consistency of the real-time hybridization assay. Mean CP values for the KNP102 samples during the assays were 27.73 ± 0.55 (n=220 assays) which corresponds to a mean calculated parasitemia of 0.0034% ± 0.0011 (range of 0.001-0.0085%). This corresponds well with previous empirical determinations of parasitemia (0.002-0.009%) in this buffalo (Sibeko et al. 2008; Papli et al. 2011), and suggests that the method for the estimation of parasitemia in buffalo blood samples is valid and that CP values between different assays may be compared.

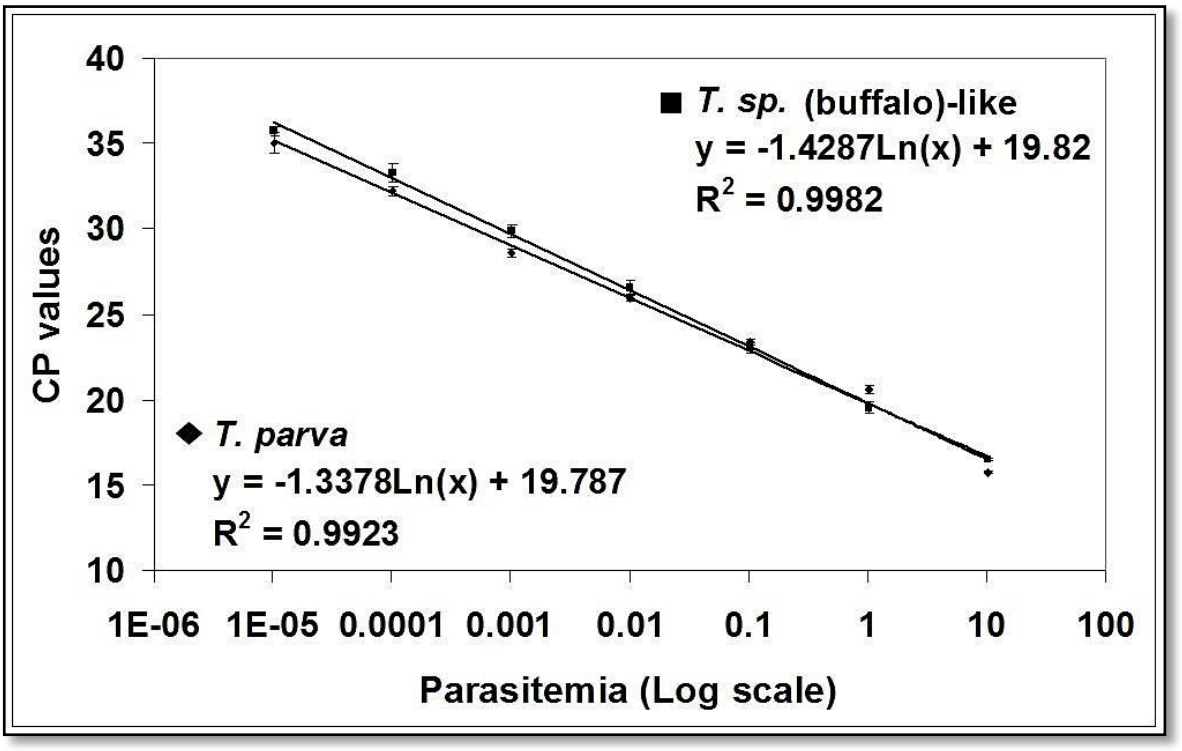


Figure 3.1: Standard curves obtained from a 10-fold serial dilution of *T. parva* and *T.* sp.(buffalo) 18S DNA templates. Indicated are the calculated parasitemia and their corresponding CP values. CP values were derived for *T. parva* and *T.* sp. (buffalo) using the 640nm and 705nm probes, respectively.

3.2.3 In-vitro simulation of mixed-infections at relevant parasitemia

Amplified PCR template solutions that represent *T. parva* parasitemia of 0.0001%, 0.001% and 0.01% were prepared using the stock solution previously used for construction of the standard curve (section 3.2.2). *T.*sp. (buffalo) PCR template was added to these to obtain 0.1-1000 fold*T.*sp.(buffalo): *T. parva* ratios and these mixed templates were analyzed using the real-time hybridization assay.

Dilution factor	Per PCR reaction (2.5ul)	Genomic equivalents/ Infected RBC	% Parasitemia	
2.25X dilution (20ul + 25ul)				
10X	= 1.0×10^{10}	= 5×10^{9}	10000	
1X	= 1.0×10^{9}	= 5×10^{8}	1000	
10^{-1}	= 1.0×10^{8}	= 5×10^{7}	100	
10^{-2}	= 1.0×10^{7}	= 5×10^{6}	10	
Realistic parasitemias				
10^{-3}	= 1.0×10^{6}	= 5×10^{5}	1	
10^{-4}	= 1.0×10^{5}	= 5×10^{4}	1.0×10^{-1}	(0.1)
10^{-5}	= 1.0×10^{4}	= 5×10^{3}	1.0×10^{-2}	(0.01)
10^{-6}	= 1.0×10^{3}	= 5×10^{2}	1.0×10^{-3}	(0.001)
10^{-7}	= 1.0×10^{2}	= 5×10^{1}	1.0×10^{-4}	(0.0001)
10^{-8}	= 1.0×10^{1}	= 5	1.0×10^{-5}	(0.00001)
10^{-9}	= 1	= 5×10^{-1}	1.0×10^{-6}	(0.000001)

The following parasitemia ranges were set up with ranges of mixed-infections.
Templates were mixed by dilution samples 10X, i.e. 10µl Tp +10 µl TspB + 80 µl water.
Thus we needed the 10X concentrate to obtain correct dilution factor.

	0.0001% Tp (10^{-7})	0.001% Tp (10^{-6})	0.01% Tp (10^{-5})
TspB:Tp	0:1	0:1	0:1
	0.1:1 (10^{-8}TspB)	0.1:1 (10^{-7}TspB)	0.1:1 (10^{-6} TspB)
	1:1 (10^{-7} TspB)	1:1 (10^{-6}TspB)	1:1 (10^{-5}TspB)
	10:1 (10^{-6} TspB)	10:1 (10^{-5}TspB)	10:1(10^{-4}TspB)
	100:1 (10^{-5} TspB)	100:1 (10^{-4}TspB)	100:1 (10^{-3} TspB)
	1000:1 (10^{-4} TspB)	1000:1 (10^{-3} TspB)	
	10000:1 (10^{-3} TspB)		

3.2.4 Reverse line blot of field and control buffalo samples

Reverse-line blot analysis was performed as described in Chapter 2. Probes for the detection of *Theileria* species in southern Africa were used (Matjila et al. 2008). These included probes for *T. annulata*, *T. bicornis*, *T. buffeli*, *T. equi*, *T. mutans*, *T. parva*, *T.*sp. (buffalo), *T.*sp. (duiker), *T.*sp. (kudu), *T.*sp. (sable), *T. taurotragi* and *T. velifera*, as well as a *Theileria/Babesia* catch-all. Samples that were negative for *T. parva* on the real-time hybridization assay were screened with RLB to identify control samples for *T. annulata*, *T. buffeli*, *T. mutans*, *T. taurotragi*, *T. velifera*, *T.*sp.(sable) and *T.*sp. (buffalo). In the case of *T.*sp. (bougasvlei) the sample was confirmed by

sequencing and submitted to Genbank (GenBank accession number: GU570997). Numbers in brackets refer to laboratory numbers used to identify diagnostic samples.

3.2.5 Conventional touch-down PCR based on protein genes

Conventional touch-down PCR based on the p67N, p67C, p104 and *Tpr* genes were performed as described in Chapter 2.

3.2.6 Locked nucleic acid based suppression of T. sp. (buffalo) template

For locked nucleic acid (LNA) suppression assays a LNA specific for *T.*sp.(buffalo) (TspB_LNA: CAGAcGgAGtTTAC-PH (Exiqon Inc.,Woburn, MA, USA)) was included in the real-time hybridization assay at a concentration of 0.5 pmol. The lower case letters indicate the position of LNA bases in the probe. This approach is based on the use of an LNA PCR clamp with a melting temperature much higher than the extension temperature used during the assay. This prevents polymerase extension and hence specific suppression of template amplification (Ren et al. 2009).

3.3 RESULTS

3.3.1 Prevalence and distribution of T. parva and T. sp. (buffalo)-like parasites

Buffalo samples (n = 6928) submitted to the PVVD laboratory for *T. parva* diagnosis during the period of 2008-2009 were analysed to determine the current geographical distribution of buffalo testing positive for *T. parva* and *T.*sp. (buffalo)-like parasites in South Africa. Approximately ~8.8% (n=609) were positive at 705 nm and indicated both *T. parva* and *T.*sp. (buffalo)-like infections. *T. parva* positive samples comprised ~3.8% (n = 261), while ~5% (n = 348) were *T.*sp. (buffalo)-like (Table 3.1). Both *T. parva* and *T.*sp. (buffalo)-like infections had the highest prevalence in Limpopo and Mpumalanga provinces, with significant overlap with the geographic range of *R. appendiculatus* (Fig. 3.2). *T.*sp. (buffalo)-like infections is also prevalent in the Free State, North-West, Eastern Cape and Kwa-Zulu-Natal Provinces. *T. parva* positive

samples were found at 22 localities, while *T*.sp. (buffalo) was found at 77 localities, indicating that the prevalence of *T*.sp. (buffalo)-like carriers is currently much higher than *T. parva*.

Table 3.1: Summary of real-time hybridization PCR results**.** Included are the total set of diagnostic samples analyzed, as well as samples from KNP and MNP. Number of samples / percentages is indicated.

	Total	***705Pos***[a]	***640Pos***[b]	**640Neg**[c]
Diagnostic	6928 / 100	609 / 8.8	261 / 3.8	348 / 5.0
KNP	262 / 100	218 / 83.2	188 / 71.7	30 / 11.4
MNP	90 /100	90 / 100	66 / 73.3	24 / 26.7

[a]Samples detected by the *Theileria* genus specific probe (705 nm positive). [b]*T. parva* positive samples (640 nm positive, 705 nm positive). [c]*T*.sp. (buffalo)-like positive samples (640 nm negative, 705 nm positive).

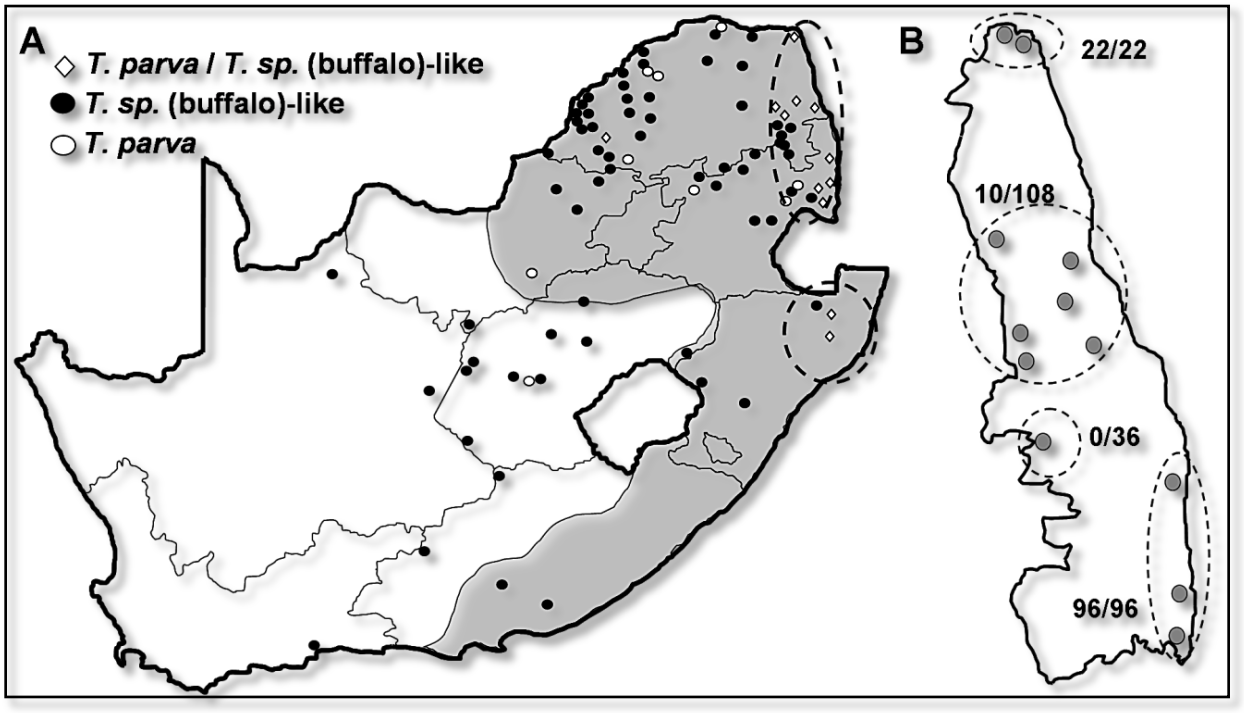


Figure 3.2: Distribution of *T. parva* and *T.* sp. (buffalo)-like samples. A) A map of South Africa indicating sites were *T. parva* and *T.* sp. (buffalo)-like samples were identified from 2008-2009. Corridor disease endemic regions are indicated by broken circles and the distribution of *R. appendiculatus* is shaded in grey (Estrada-Peña, 2003). The upper broken circle indicates the approximate position of the Kruger National Park. B) The presence of *T.* sp. (buffalo) in the

Kruger National Park. Shaded circles indicate sites where buffalo were sampled and the numbers sampled from larger areas are indicated as well as the number that tested positive for *T.* sp. (buffalo) on RLB analysis.

3.3.2 Parasitemia ranges for T. parva and T. sp. (buffalo)-like parasites in buffalo

Competitive PCR suppression may occur if the parasitemia levels of *T.*sp. (buffalo)-like organisms are similar or higher than that of *T. parva* in carrier animals. Parasitemia levels were estimated from a calculation based on standard curve analysis derived from real-time PCR CP values (Fig. 3.1). This indicated that parasitemia for *T. parva* and *T.*sp. (buffalo)-like parasites is similar in buffalo, ranging from 0.000001-1%, with the majority of samples (>90%) falling between 0.0001% and 0.1% (Fig. 3.3). In addition, the frequency distribution curves would suggest that *T.*sp. (buffalo)-like samples are pre-disposed towards higher parasitemia, relative to that of *T. parva* within the 0.0001-0.1% range (Fig. 3.3). In mixed infections, *T.*sp. (buffalo)-like parasites could potentially be present at ratios of up to 1000:1 compared to *T. parva.*

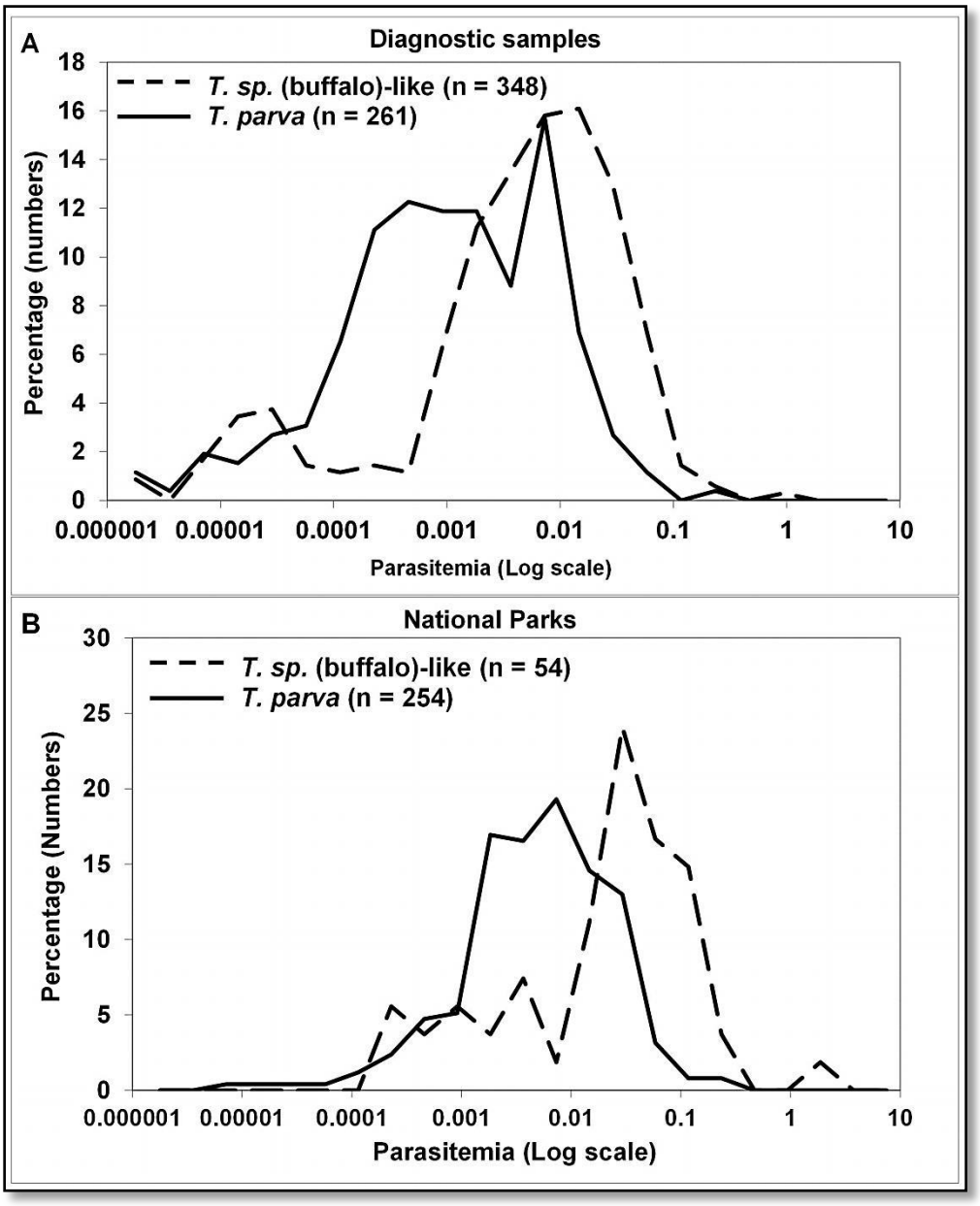


Figure 3.3: Frequency distribution of estimated parasitemia for *T. parva* and *T.* sp. (buffalo)-like parasites in field samples from African buffalo. A) Parasitemia calculated for diagnostic samples. B) Parasitemia calculated for National Parks samples. Parasitemia was calculated from CP values obtained from real-time PCR data for *T. parva* (640nm) and *T.* sp. (buffalo)-like (705nm) samples using standard curves. The number of samples for each sample type analyzed is indicated and the percentage of the total number was determined by grouping parasitemia in 2-fold decreasing bins.

3.3.3 The suppressive effect of mixed infection on real-time PCR

Mixed infections was simulated by combining *T. parva* (0.0001%-0.01%) with *T.*sp. (buffalo) DNA template at ratios ranging from 0.1:1-1000:1 *T.*sp. (buffalo) vs. *T. parva* (Fig. 3.4). *T. parva* signal (640 nm) was notably suppressed in the lower parasitemia range (0.0001-0.001%), when *T.*sp. (buffalo) was present at ratios of 100:1 and higher (0.01-0.1%). At higher *T. parva* parasitemia (0.01%), the suppression effect was not observed at the highest *T.*sp. (buffalo) ratio used (10:1) that corresponds with 0.1% parasitemia. However, at a ratio of 100:1 (1% *T.*sp. (buffalo)) suppression was still observed (results not shown), although the incidence of this level of parasitemia is not high within field samples (Fig. 3.3).

3.3.4 Mixed infections in field populations

In Chapter 2 and the previous sections the questions were raised whether mixed infections occur in the field and whether PCR suppression is present in these populations. Buffalo samples from two National Parks (KNP and MNP) were characterized for which 262 and 90 samples, respectively, were analyzed for mixed infections of *T. parva* and *T.* sp. (buffalo)-like parasites. In both parks ~70% of all samples were *T. parva* positive using the real-time hybridization assay (Table 3.1). Buffalo negative for *T. parva*, but positive for *T.* sp. (buffalo)-like parasites showed a prevalence of ~10 and 26% in KNP and MNP, respectively. The frequency distribution of the parasitemia for *T. parva* and *T.* sp. (buffalo)-like samples follows a similar trend compared to the diagnostic samples (Fig. 3.3B). *T.* sp.(buffalo)-like samples are, however, still predisposed towards the higher end of the parasitemia range, to such an extent that ratios of 10:1 or 100:1 might be possible at lower *T. parva* levels.

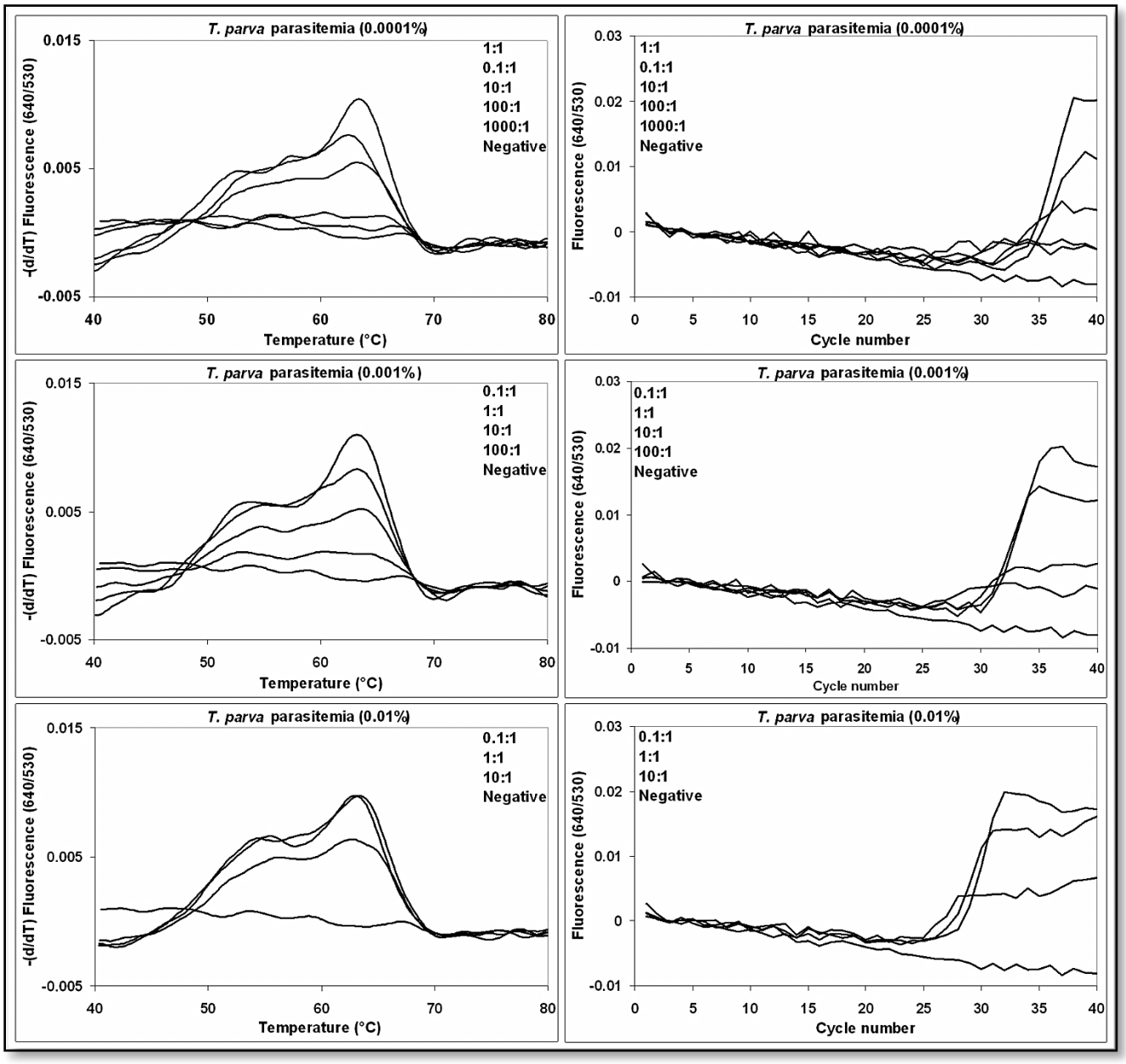


Figure 3.4: Simulation of *T. parva* and *T.*sp. (buffalo) mixed-infections at parasitemia ≤0.1%. Indicated are *T. parva* DNA templates at various calculated parasitemia (0.0001-0.01%), mixed with *T.*sp. (buffalo) template (0.001-0.1%) at ratios from 0.1-1000 *T.*sp. (buffalo): *T. parva*. Indicated are melting peaks and amplification curves for the 640nm channel.

T. sp. (buffalo), *T. mutans* and *T. velifera* were present in all Marakele samples as determined by RLB analysis (Table 3.2). In contrast, RLB analysis could only detect 64% *T. parva* positive samples. In the case of the KNP, larger variations with regard to mixed infections were observed by RLB analysis, with 88% and 71% being positive for *T. mutans* and *T. velifera* and only 31% being *T. parva* positive (Table 3.2). In the case of *T.* sp. (buffalo) only 49% were positive by RLB analysis. The majority of the *T.* sp. (buffalo) samples originated from northern and south-eastern parts of the KNP, with a limited prevalence in the central regions (Fig. 3.2B).

Table 3.2: Summary of RLB results from KNP and MNP**.** Indicated is the number of buffalo found positive with percentages in brackets.

Samples	***Total (n= 352)***	***T. buffeli***	***T. mutans***	***T. parva***	***T.*sp. (buffalo)**	***T. velifera***
KNP	262 (100)	0 (0)	231 (88)	81 (31)	128 (49)	187 (71)
MNP	90 (100)	8 (9)	90 (100)	58 (64)	90 (100)	89 (99)

3.3.5 Detection of T. parva based on protein gene markers

All conventional protein gene PCR's showed a good correlation with the real-time hybridization test for *T. parva* positive samples (Table 3.3). All samples negative (no signal at 705 nm) on the hybridization test were also negative on the protein genes and most of these had infections of *T.*sp. (buffalo), *T. mutans* and *T. velifera* as indicated by RLB analysis (Table 3.3). In the case of the *T. parva* positive samples, higher than 90% correlation were obtained between the hybridization, p67N, p67C and p104 genes. In the case of the *Tpr* gene, all samples derived from MNP were negative for *T. parva* and hence the low correlation. For the *T.*sp. (buffalo)-like samples the correlation was ~43-50% for p67N, p67C and p104 and 74% for *Tpr* due to the negative status of the MNP samples based on this gene, while correlation between p67N, p67C and p104 was 89-93%. This indicated that ~57% of the samples in the test group (31 samples) that were negative on the hybridization assay were positive for *T. parva* with three different

protein gene markers. This also confirmed that the same PCR suppression observed in the KNP occurs in Marakele National Park.

Table 3.3: Correlation between different PCR tests for the National Parks samples**.**

Sample status on hybridization assay (Number of samples)	***P67N***	***P67C***	***P104***	***Tpr***
Negative (42)	42 [a]	42 [a]	42 [a]	42 [a]
640POS/ 705POS (239)	222[b]	235 [b]	235 [b]	168 [b]
40NEG / 705 POS (54)	27[c] 27[d]	25[c] 29[d]	23[c] 31[d]	40[c] 14[d]

[a]Samples that tested negative on the hybridization assay and protein genes. [b]Samples that tested positive for *T. parva* (640 nm positive, 705 nm positive) on the hybridization assay and protein genes. [c]Samples that tested negative for *T. parva* but positive for *T*.sp. (buffalo)-like (640 nm negative, 705 nm positive) on the hybridization assay and negative for *T. parva* on the protein genes. [d]Samples that tested negative for *T. parva* but positive for *T*.sp. (buffalo)-like (640 nm negative, 705 nm positive) on the hybridization assay and positive for *T. parva* on the protein genes.

3.3.6 Locked nucleic acid suppression of T. sp. (buffalo) in simulated mixed infections

Incubation of the *T*.sp. (buffalo) template with an LNA clamp specific for *T.* sp. (buffalo)showed that amplification was suppressed by an average of six cycles (Fig. 3.5). This corresponds to a decrease in parasitemia of ~100-fold. The LNA had no effect on amplification of *T. parva* (results not shown). When LNA was included in the mixed infection simulation, *T. parva* could be detected in all cases where 100:1 fold excess of *T*.sp. (buffalo) previously caused suppression (Fig.3. 4 and Fig. 3.5B and Fig. 3.5C).

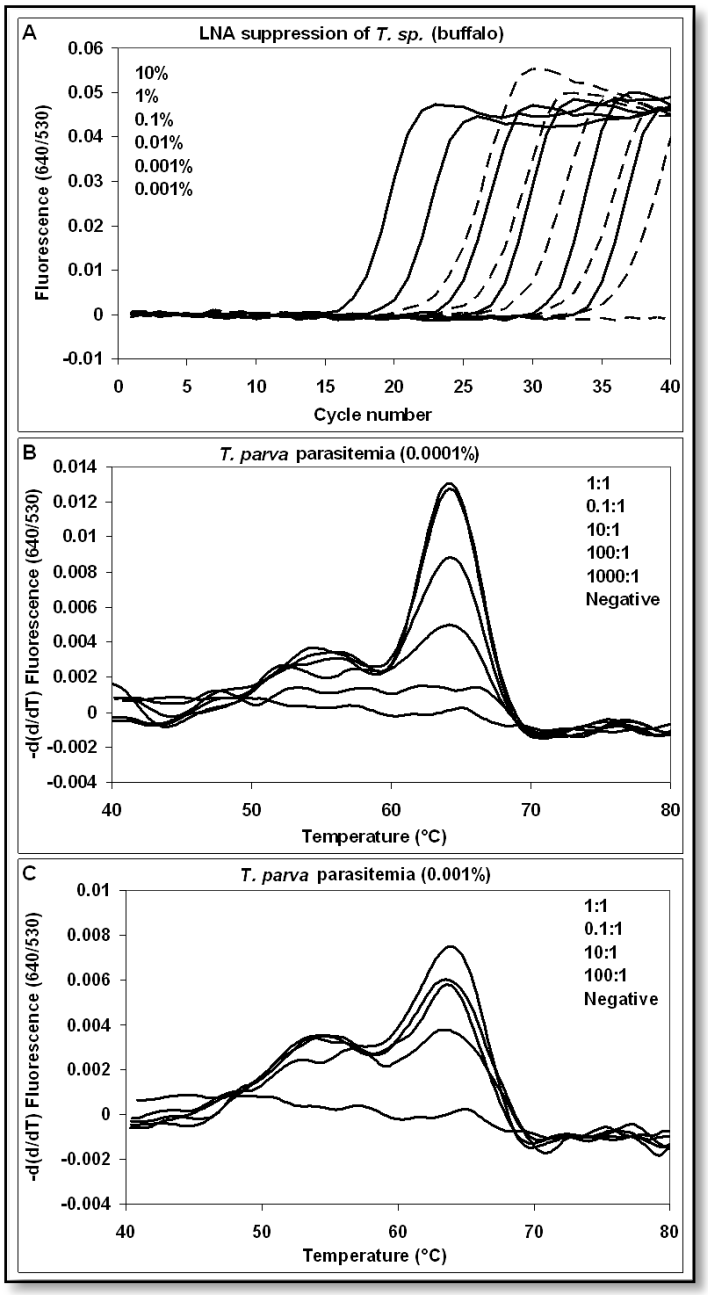


Figure 3.5: LNA-based suppression of *T*.sp. (buffalo) in simulated mixed infections. A) Suppression of *T*.sp. (buffalo) amplification by the *T. sp.* (buffalo) specific LNA. Indicated is *T*.sp. (buffalo) template without (solid line) and with(broken line) added LNA. Calculated parasitemia correlating with high to low cycle numbers are indicated. Mixed infection simulation of *T. parva* and *T*.sp. (buffalo) at *T. parva* parasitemia of 0.0001% (B) and 0.001% (C) in the presence of *T*.sp. (buffalo) at various ratios indicated (top to bottom) in the presence of LNA.

3.3.7 Locked nucleic acid suppression in buffalo samples

The set of 31 samples that tested negative for *T. parva* using the real-time hybridization assay, but positive for the protein gene PCR's were re-tested with added LNA (Fig. 3.6). The melting curves without LNA added show typical *T.*sp. (buffalo)-like profiles that are ambiguous to interpretation (Fig. 3.6A). In the presence of LNA, the profiles resolve to a *T. parva* positive status (Fig. 3.6B), indicating that *T.*sp. (buffalo) suppression occurred in these samples.

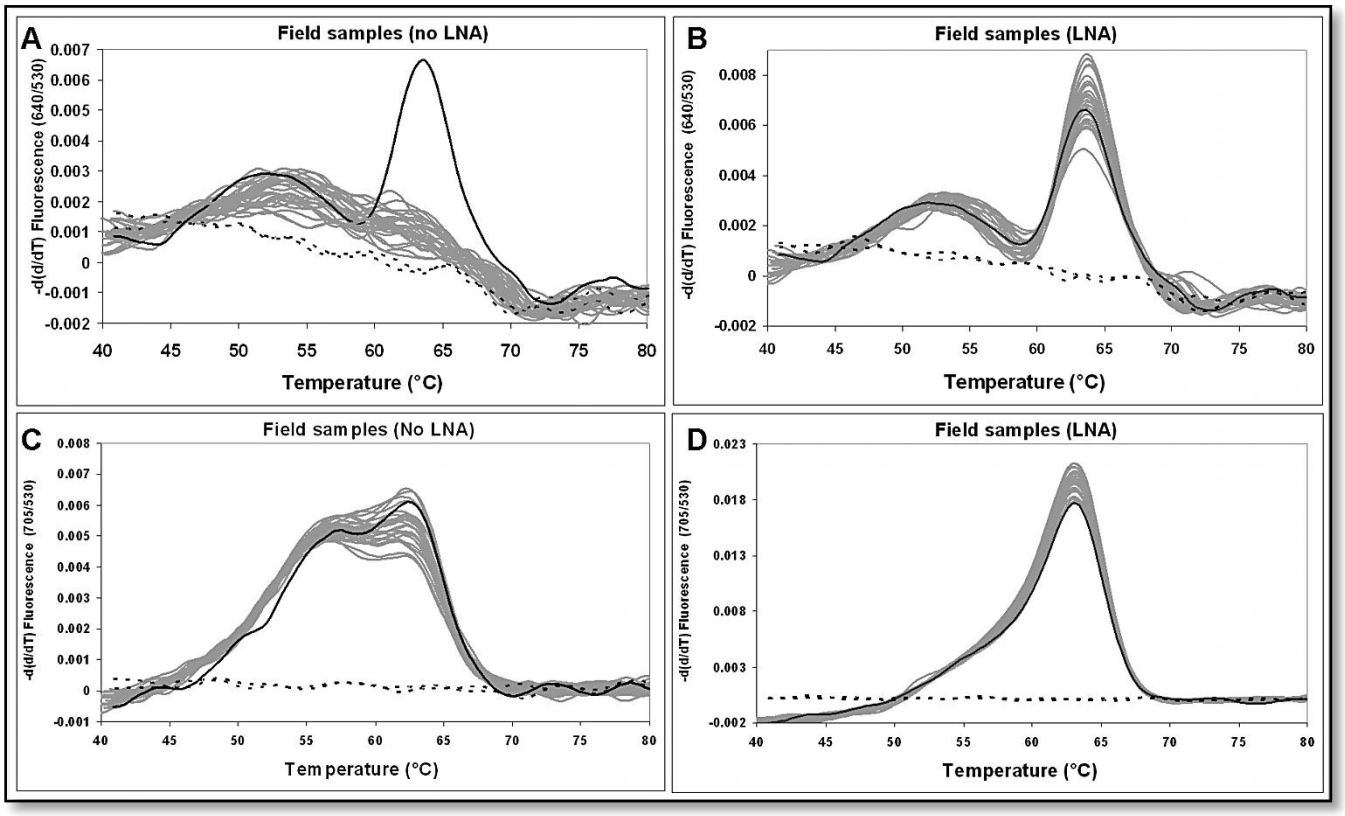


Figure 3.6: LNA-based suppression in buffalo samples. Normal hybridization conditions are indicated on the left and the same samples with added LNA on the right, while the 640nm and 705nm channels are indicated at the top and bottom, respectively. Grey lines correspond to field samples, the solid black line to the *T. parva* positive control and the broken black lines to negative controls.

3.3.8 Potential for mixed infections within diagnostic samples

The question is raised on the extent of *T.*sp.(buffalo)-like suppression that occurs in diagnostic samples. The diagnostic sample set analysed in this study was also screened with the p67N PCR to detect possible false-negative samples. A correlation of 94% was obtained between the real-time hybridization and p67N test for 603 samples screened (230 *T. parva* positive, 339 *T.*sp. (buffalo)-like positive). Thirty-three samples (5%) were negative on p67N, but positive on the hybridization test indicating that genotypic variations do occur in the primer regions of p67N for a limited number of samples. Only one sample was positive with p67N and negative for *T. parva* using the hybridization test. This sample was re-tested with all the protein genes as well as the hybridization test with added LNA and was confirmed to be positive for *T. parva*. The only other sample of a mixed infection submitted for diagnosis (outside of the dataset analysed) that showed suppression was a buffalo (28698) from the Corridor disease endemic region. This sample tested positive on all protein gene based PCR's as well as with the added LNA hybridization assay.

3.4 DISCUSSION

Cape buffalo from southern Africa can harbor *T. buffeli*, *T. mutans* and *T. velifera*, as well as *T. parva*, *T.*sp. (buffalo) and *T.*sp. (bougasvlei) (Allsopp et al. 1999; Zweygarth et al. 2009). This makes accurate diagnosis at species level problematic using conventional approaches such as light microscopy or IFAT. DNA blotting and probe approaches that use universal *Theileria* primer sets, such as RLB or slot blotting have limited use for accurate diagnostics, as PCR competition in the case of mixed infections affect sensitivity (Allsopp et al. 1993; Gubbels et al. 1999). Slot-blot hybridization analysis indicated that 33% of buffalo from KNP were *T. parva* positive, even though 100% was positive in the IFAT (Allsopp et al. 1999). This lack of correlation was confirmed in the current study, where the National Parks buffalo samples tested with RLB found only 27-64% of samples positive compared to ~70% with the real-time hybridization assay. Even so, the protein gene assays in the current study detected an additional 10% *T. parva* positive cases. The RLB analysis also indicated that buffalo from the National

Parks are infected with three or more *Theileria* spp., including *T.*sp. (buffalo). Allsopp et al. (1999) found *T.*sp. (buffalo) in 54% of buffalo samples from "northern" regions of the KNP which correlate with results from the current study.

Parasitemia levels for *T. parva* and *T.* sp. (buffalo)-like parasites have previously not been estimated to the extent described in this study. The ranges observed for *T. parva* (0.0001-0.1%) correlate well with those of buffalo-adapted *T. parva* in Cape buffalo and cattle with low piroplasm levels (<0.1%) in carrier animals (Brocklesby and Barnett, 1966; Barnet and Brocklesby, 1966a; Barnet and Brocklesby, 1966b; Neitz, 1958). The frequency distribution ranges suggest that most *T. parva* samples from buffalo (>98%) should be readily detectable using the real-time hybridization assay, while the rest of the samples lie at the lower range of detection for real-time PCR. The sensitivity of the current hybridization test to detect *T. parva* in carrier buffalo is therefore adequate, although it should be noted that a few infected animals might not be detected. This correlates with observations on the East Coast fever carrier state in cattle from Kenya, where a PCR based on the p104 gene was sensitive up to 1.4 parasites/µl of infected blood ($2.8E^{-5}$% parasitemia~similar to the hybridization assay), but failed to detect 4.6% of infected animals detected by a subsequent nested PCR (Odongo et al. 2010). Whether animals with such low parasitemia (<E^{-6}%) can effectively infect ticks remains to be determined. The possibility of higher false-positives rates using nested PCR should be considered. The latter study also found the prevalence of East Coast fever in asymptomatic cattle from endemic regions in Kenya to range from 37-42% (Odongo et al. 2010). This is significantly lower than the Corridor disease prevalence found in carrier buffalo using the hybridization assay (~71%), p67N (~74%), p67C (~85%) and p104 (~79%) genes. This could indicate that the prevalence of *T. parva* parasites in carrier cattle is lower than in buffalo. However, previous studies have indicated that in endemic situations the majority of cattle were exposed to *T. parva* and would presumably be carriers in a situation analogous to buffalo (Young et al. 1978; Young et al. 1986). Alternatively, the p104 primer sets used for amplification might not detect all sequence variants for this protein gene, as was observed in the present study for all of the protein genes.

The MNP samples all tested negative for *T. parva* using the *Tpr* gene. The *Tpr* gene occurs as a multi-copy locus (24 copies on chromosome 3) in the genome of *T. parva* with a variable 5'-end and a highly conserved 3'region (Gardner et al. 2005). This conserved region codes for

trans-membrane regions and has been suggested to be maintained by concerted evolution (Bishop et al. 1997). Any nucleotide substitutions in this region would be rapidly homogenized, so that rapid divergence of these sequences might occur in geographically isolated populations. Introduction of *T. parva* into MNP which is outside the endemic area for *T. parva* probably occurred as a single event that then spread throughout the resident buffalo population. A founder effect coupled to sequence variation in the *Tpr* primer areas (due to concerted evolution), could explain the lack of any positive samples in MNP with this gene.

*T.*sp. (buffalo) was originally identified in buffalo from East Africa and distinguished from *T. parva* based on differences in antigenicity and subsequent sequencing of its 18S rRNA gene (Allsopp et al. 1993; Conrad et al. 1987). This parasite was cultured *in vitro* and significant antibody cross-reactivity was observed with *T. parva* (Conrad et al. 1987). This might explain the observation that free-ranging buffalo tested previously were 100% positive on IFAT for *T. parva* (Allsopp et al. 1993). In addition its close resemblance to *T. parva* in the 18S rRNA raised the question whether this was a variant strain of *T. parva* (Allsopp et al. 1993; Conrad et al. 1987). Subsequently, this parasite was detected in buffalo from the KNP that showed that *T.*sp. (buffalo) were detected in 54% of animals compared to the 33% that was *T. parva* positive (Allsopp et al. 1999). This corresponds to results from the current study that showed that *T.*sp. (buffalo)-like parasitemia and their prevalence in buffalo are on average higher than observed for *T. parva*. The establishment of a *T.*sp. (buffalo) cell line from a buffalo of South African origin showed that on 18S level it is identical to the parasite described from East Africa (Zweygarth et al. 2009). *T.*sp. (bougasvlei) is a closely related parasite described in the latter study. Circumstantial evidence (sentinel cattle in contact with carrier buffalo) would suggest that both parasites are not infective to cattle (F.T.Potgieter, unpublished observation).

The tick vectors and the geographic ranges for both parasites are unknown. Ticks found on buffalo in KNP indicated that potential vectors could be *Amblyomma hebraeum, A. marmoreum, Hyalomma truncatum, R. appendiculatus, R. evertsi evertsi* or *R. simus* (Horak et al. 2007). The distribution patterns for these ticks largely overlap with that of *R. appendiculatus* in the North-West and Northern Provinces, as well as Mpumalanga, Kwa-Zulu Natal and the eastern parts of Eastern Cape (Estrada-Peña, 2003; Horak et al. 2006a; Walker et al. 2000). If any of these ticks are vectors, mixed-infections will be common.

The limited distribution of *T*.sp. (buffalo) in the northern and south-eastern regions of the KNP poses an interesting conundrum. All the tick species mentioned above were distributed across the KNP (Spickett et al. 1991; Horak et al. 2006b). It could therefore be expected that *T*.sp. (buffalo) has a wider distribution if one of the above mentioned ticks were its vector. Tick burden (and numbers of infected ticks) might differ in various geographical areas due to the prevalence of infected buffalo and other competing non-infected vertebrate hosts. This was the case for *A. hebraeum* and *R. evertsi evertsi* in central and southern regions of the KNP, where buffalo, zebra and impala numbers influenced tick prevalence (Horak et al. 1995).

Suppression of the *T. parva* positive signal by the presence of *T*.sp. (buffalo) template at concentrations relevant for field parasitemia has been shown to occur *in vitro*. Evidence was also provided that this happens in free-ranging buffalo. Thus far the extent to which this occurred during routine diagnostics has been minimal (<0.5%). This is mainly due to the low incidence of *T. parva* and *T*.sp. (buffalo)-like samples submitted for routine diagnostics (<10%), with the correlated low probability of finding mixed infections in such samples. The number of *T. parva* free buffalo with *T*.sp. (buffalo)-like infections will probably increase in the future, as movement of such buffalo are not restricted by current legislation. This is reflected by the higher prevalence of *T*.sp. (buffalo)-like carrier animals at various sites identified in this study. Buffalo could remain life-long carriers and where the vector for *T*.sp. (buffalo) is present would also lead to its spread among buffalo populations. The geographic distribution data for *T. parva* and *T*.sp. (buffalo)-like parasites also suggests that their respective vectors overlap geographically and that mixed infections can be expected to occur. Introduction of *T. parva* into a buffalo herd already infected with *T*.sp. (buffalo), could as such, potentially lead to cases which will not be detected using the current test. In an endemic situation where both *T. parva* and *T*.sp. (buffalo) occur, the current data suggest that at least 10% of *T. parva* infections might be misdiagnosed. While this might not be a problem of immediate concern, more sensitive tests able to accurately discriminate between *T. parva* and *T*.sp.(buffalo)-like parasites, which is not affected by mixed-infections will be required in the future (See Chapter 4).

In the case of cattle, the effect of mixed *T*.sp.(buffalo)-like infections on the hybridization test does not seem to be problematic, as out of ~2500 diagnostic samples none has presented a

T.sp. (buffalo)-like profile (unpublished observation). This would imply that *T*.sp.(buffalo) and *T*.sp. (bougasvlei) do not infect cattle.

Mixed infections of *T. parva* and *T*.sp. (buffalo) can affect the diagnostic sensitivity of the hybridization PCR assay of Sibeko et al. (2008). In cases where this is suspected the current test could be supplemented by PCR assays based on protein genes developed in Chapter 2. In addition, the use of an LNA which is able to suppress *T*.sp. (buffalo) can be incorporated into the test if mixed infections are suspected, or new diagnostic tests not affected by mixed-infections can be implemented.

CHAPTER 4

The Hybrid II assay: A sensitive and specific real-time hybridization assay for the diagnosis of Theileria parva infection in Cape buffalo (Syncerus caffer) and cattle*

**(Work presented in this chapter was accepted for publication in Parasitology. Pienaar et al. 2011b)*

4.1 INTRODUCTION

Corridor disease, East Coast fever and January disease (Zimbabwe theileriosis) are syndromes caused by *Theileria parva*. Infections result in a lymphoproliferative pathology that is associated with high mortality in cattle (Norval et al. 1992). Historically *T. parva* was classified into 3 subspecies based on biological and clinical differences namely, Corridor disease (*Theileria parva lawrencei*), East Coast fever (*Theileria parva parva*) and January disease (*Theileria parva bovis*) (Uilenberg, 1976; Lawrence, 1979). Serological cross-reaction and genetic similarity between the various subspecies has led to the abolishment of this classification system, with *T. parva* being currently distinguished by their host origin as either cattle- or buffalo-adapted (Norval et al. 1991). East Coast fever and January disease are caused by transmission between carrier and susceptible cattle (Young et al. 1986; Koch et al. 1992). In contrast, Corridor disease occurs when *T. parva* is transmitted from carrier Cape buffalo (*Syncerus caffer*) to cattle (Neitz et al. 1955; Neitz, 1957). While cattle carrier states have been shown to occur under laboratory conditions for Corridor disease, no carrier state has been confirmed for cattle under field conditions in South Africa (Neitz, 1958; Barnett and Brocklesby, 1966b; Potgieter et al. 1988). Concerns do, however, exist that buffalo-derived *T. parva* could establish itself in a carrier state in cattle that can lead to a situation similar to that found in East Africa with East Coast fever (Potgieter et al. 1988; Yusufmia et al. 2010). In 1902, East Coast fever was introduced from East Africa into South Africa (Theiler, 1904), but was eradicated by a strict quarantine, systematic dipping and slaughter campaign of affected cattle by 1956 (Neitz, 1957). As such, East Coast fever is still present in East and southern Africa, but is considered to be absent in South Africa (Potgieter et al. 1988; Sibeko et al. 2010). Corridor and January disease were first identified in Zimbabwe, with the latter never considered present in South Africa (Neitz, 1957; Potgieter et al. 1988). Corridor disease was first recognized in South Africa in the corridor formed between the historical Hluhluwe and Umfolozi game parks, hence the name (Neitz et al. 1955). The current endemic regions in South Africa include the Hluhluwe-Imfolozi Park (Kwa-Zulu Natal), the Kruger National Park (KNP) and regions between and surrounding these areas (Potgieter et al. 1988). *Rhipicephalus appendiculatus*, the main tick vector is, however, widespread across the North-West and Northern Provinces, as well as Mpumalanga, Kwa-Zulu Natal and the eastern parts of Eastern Cape (Estrada-Peña, 2003). *R. zambeziensis*, another important vector has a

limited geographical range in the north-eastern and western regions of the Northern Province (Walker et al. 1981; Lawrence et al. 1983). The geographical range of *T. parva* will expand if infected vector ticks or carrier buffalo are introduced into non-endemic regions of South Africa. Themovement of buffalo outside the endemic disease regions is therefore strictly regulated by the Department of Agriculture, Forestry and Fisheries (Animal Disease Act 1984, Act No. 35), to prevent disease outbreaks among cattle, that is totally naïve to *T. parva* in South Africa (Thompson et al. 2008). This is mainly to prevent disease outbreaks among cattle and the potential establishment of a carrier state in cattle that will lead to a situation that resembles that of the original East Coast fever epidemic (Yusufmia et al. 2010). 'Disease free' buffalo have become a lucrative commodity due to the expansion of the eco-tourism trade in South Africa and translocation of buffalo depends on their disease-free status (Collins et al. 2002; Thompson et al. 2008). Buffalo are tested for *T. parva* infection by serology (indirect fluorescent antibody test) and real-time hybridization PCR before translocation (Burridge and Kimber, 1972; Sibeko et al. 2008). It was recently shown that up to 10% of free-ranging buffalo from National Parks with mixed infections of *T. parva* and *T.* sp. (buffalo) could be misdiagnosed due to suppression of PCR using the real-time hybridization test (Pienaar et al. 2011a). Ambiguous results, difficult to interpret, are occasionally found for*T. parva*-negative samples that are positive for *T.* sp. (buffalo)-like parasites (Pienaar et al. 2011a). The latter parasite has not been shown to be infective to cattle or to be pathogenic and is not a concern for the Veterinary Authorities in South Africa (Mans et al. 2011a; Pienaar et al. 2011a). As such, more accurate and specific assays are needed for accurate diagnosis of *T. parva* in carrier buffalo. The current study describes the Hybrid II assay, an improved real-time hybridization PCR assay that is not affected by mixed-infections of *T.* sp. (buffalo)-like parasites and *T. parva*.

4.2 MATERIALS AND METHODS

4.2.1 Collection of blood samples, DNA extraction and real-time hybridization assay

Buffalo and cattle samples submitted to the Parasites, Vectors and Vector-Borne Diseases (PVVD) laboratory during 2008-2011 for routine *T. parva* diagnosis were processed for analysis as described in Chapter 2. Genomic DNA was extracted and the real-time hybridization PCR

performed and analyzed as described in Chapter 2. Based on criteria for the real-time hybridization test described in Chapter 2, diagnostic samples analysed by the hybridization real-time PCR assay were selected for analysis in this study that included 525 negative samples, 860 *T. parva* positive samples (689 buffalo and 171 cattle) and 1036 *T.*sp. (buffalo)-like positive samples.

4.2.2 Design of the Hybrid II assay

A *Theileria* genus specific forward (TgF: GGTAATTCCAGCTCCAATAG) and a *T. parva* specific reverse primer(TpR: AAAGTAAACATCCAGACAAAGCG), referred to as the Hybrid II primer set were designed to amplify a 145 bp fragment of the V4 hypervariable region from the 18S rRNA gene (Fig. 4.1). The hybridization probe pair previously used for detection of *T. parva* (Sibeko et al. 2008), was used to detect *T. parva* at 640 nm.

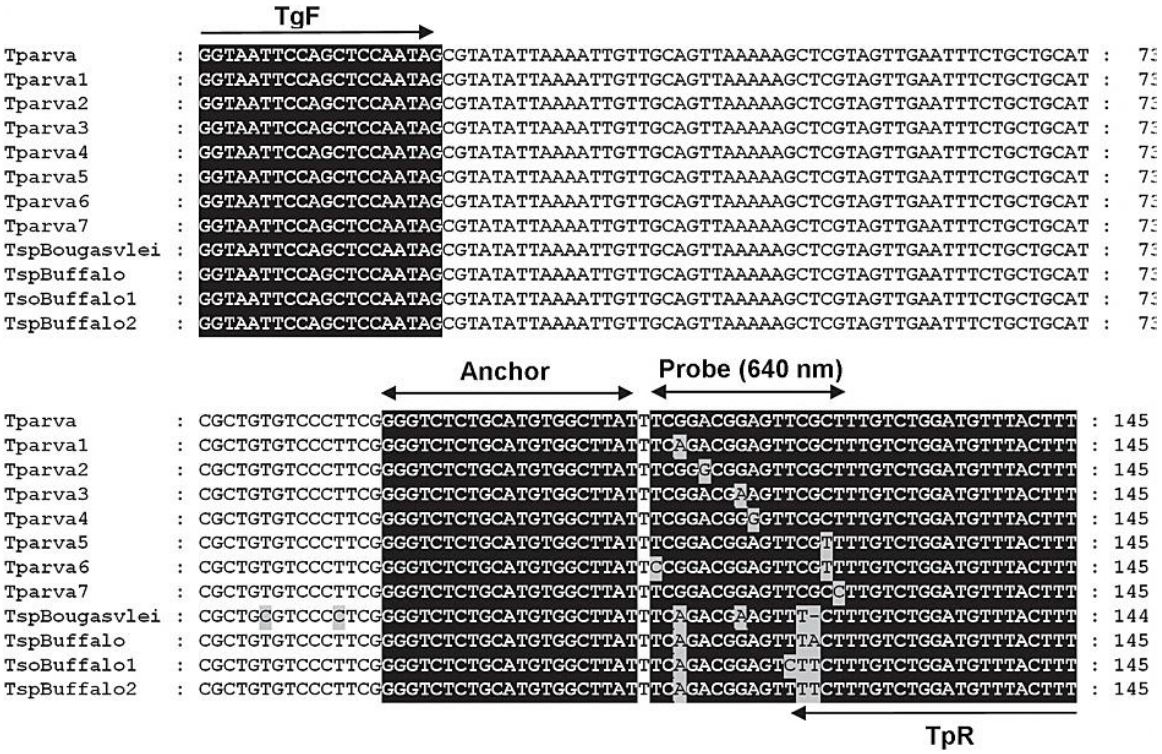


Figure 4.1: Summary on the design of the Hybrid II assay. Indicated is the 145 bp region amplified from the V4 hyper-variable region of the 18S rRNA. *Theileria* genusspecific (TgF) and *T. parva* specific reverse (TpR) primer as well as the anchor and probe regions are marked with arrows and dark shading. Differences between sequences are shaded in gray. The closest related sequences to *T. parva* is included (*T.* sp. (buffalo) and *T.* sp. (bougasvlei)) as well as variants of *T. parva* accoding to Mans et al. (2011a).

4.2.3 Optimization of the Hybrid II assay conditions

The Hybrid II assay was initially developed using 4µl LightCycler-FastStart DNA MasterPlus Hybridization mix (Roche Diagnostics, Mannheim, Germany), or 4µl LightCycler® 480 Genotyping Master mix (Roche Diagnostics, Mannheim, Germany). For samples that was positive, an amplification curve was obtained at 640nm and a melting peak at ~63°C (Fig. 4.2). Low fluorescence signal was observed for the LightCycler-FastStart DNA MasterPlus Hybridization mix, while sensitivity was affected with the LightCycler® 480 Genotyping Master mix (Fig. 4.2 and Fig. 4.3).

Subsequently, the Hybrid II assay was optimized using 2µl of each of the above and designated as the Hybrid II assay mix. Reaction conditions included in all cases 1U uracil deoxy-glycosylase (UDG) (Roche Diagnostics, Mannheim, Germany), 0.5pmol forward (TgF: GGTAATTCCAGCTCCAATAG) and reverse primer (TpR: AAAGTAAACATCCAGACAAAGCG), 0.1pmol each of the *T. parva* (LC640) hybridization anchor and probe pairs at a final volume of 20µl. Reaction conditions included an initial UDG activation step at 40°C (10 min), followed by a pre-incubation step at 95°C (10 min). An initial 10 cycles of denaturation (95°C, 10s), annealing (60°C, 10s) and extension (72°C, 15s) were followed by a touch-down procedure from 60-56°C over fifteen cycles, followed by 20 cycles at 56°C. Melting curves were obtained using a ramp rate of 0.2°/s from 40-95°C.

These conditions were used on both Roche LightCycler® 2.0 and LightCycler® 480 systems. For all assays the gold standard positive (KNP102) and negative (9426) controls used for routine diagnostics were included. KNP102 is a *T. parva* positive carrier buffalo that was previously used as gold standard positive control (Sibeko et al. 2008). The negative control was born and raised in a herd that has been under quarantined tick-free conditions for several decades.

4.2.4 Specificity of Hybrid II assay

Buffalo or cattle samples that tested negative with the hybridization assay for *T. parva* were analysed by reverse line blot analysis (Gubbels et al. 1999; Pienaar et al. 2011a). This identified

samples that possessed 18S template for *Babesia bigemina* and *B. bovis,Theileria annulata*, *T*.sp. (duiker), *T*.sp. (kudu*)* and*T*.sp. (sable). The 18S gene for various *Theileria* species was also amplified, cloned and sequenced (Mans et al., 2011a) and this identified samples with 18S template for *T. buffeli*-like C, *T. buffeli* type D-like, *T. mutans*, *T. mutans* like-1, *T. mutans* like-2, *T. mutans* like-3, *T. mutans* MSD, *T*.sp. (bougasvlei), *T*.sp. (sable-like), *T. taurotragi, T. velifera*, *T. velifera*-like A and *T. velifera*-like B. Cattle samples positive for the *Trypanosoma* spp. *T. vivax, T. congolense* Savannah and *T. congolense* Kilifi were confirmed by cloning and sequencing of the 18S gene (Mamabolo et al. 2009).

4.2.5 Sensitivity of the Hybrid II assay

A quantified 18S *T. parva* template obtained from a purified 1100 bp PCR product (10-fold serial dilution [Section 3.2.2]) (Pienaar et al. 2011a) was used to determine the analytical sensitivity of both the Hybrid II and hybridization assays using procedures described above. Sensitivity was also determined using the *T. parva* positive gold standard control, buffalo KNP102 (Sibeko et al. 2008). For this, a tenfold dilution range was prepared in triplicate using frozen EDTA blood previously collected from KNP102 and negative cattle control EDTA blood before extraction and testing. The parasitemia of KNP102 was determined previously for this batch of blood (Sibeko et al. 2008; Papli et al. 2011). Parasitemias were calculated for the Hybrid II and hybridization assays using the linear regression curves obtained from the defined 18S template used to assess analytical sensitivity, as previously described in section 3.2.2 (Pienaar et al., 2011a).

4.2.6 Supplementary PCR assays for T. parva

PCR assays using primers specific for the p67N, p67C and p104 gene fragments of *T. parva* were performed as previously described in Chapter 2 and Chapter 3 (3.2.5) (Pienaar et al. 2011a). To suppress the *T*.sp. (buffalo) DNA template concentration in selected diagnostic

samples, a locked-nucleic acid (LNA) specific for *T.*sp. (buffalo) were included in the hybridization assay as previously described in Chapter 3 (3.2.6) (Pienaar et al. 2011a).

4.2.7 Simulation of mixed-infections of T. parva and T. sp. (buffalo)

Mixed-infections were simulated as described previously in Chapter 2 (3.2.3) (Pienaar et al., 2011a). Briefly, defined PCR templates for *T. parva* that correspond to parasitemias of 0.0001% and 0.001% were mixed with *T.*sp. (buffalo) template at ratios that ranged from 0.1:1 to 10000:1. The mixes were then used as templates for Hybrid II assays.

4.2.8 Detection of T. parva variants

Samples previously shown to harbour variant 18S sequences of *T. parva* (Mans et al. 2011a) were tested using the Hybrid II assay.

4.3 RESULTS

4.3.1 Comparison of various real-time PCR mixes

The Hybrid II assay combined equal volumes of both the LightCycler® FastStart DNA Master^{PLUS} HybProbe and LightCycler® 480 Genotyping Master mixes as it was found that this gave more stable amplification curves and melting peaks than the LightCycler® FastStart DNA Master^{PLUS} HybProbe mix and showed higher sensitivity than the LightCycler® 480 Genotyping Master mix, respectively (Fig. 4.2 and Fig. 4.3). It is less prone to the hook effect and allows for the use of both Roche LightCycler® 2.0 and LightCycler® 480 systems, with comparable results (Fig. 4.2 and Fig. 4.3). The Hybrid II assay therefore describes a hybrid between these two different mixes and instruments. It should be noted that a shoulder peak was observed at ~52°C (Fig. 4.2 and Fig. 4.3), which was also observed with the previous test (Sibeko et al. 2008).

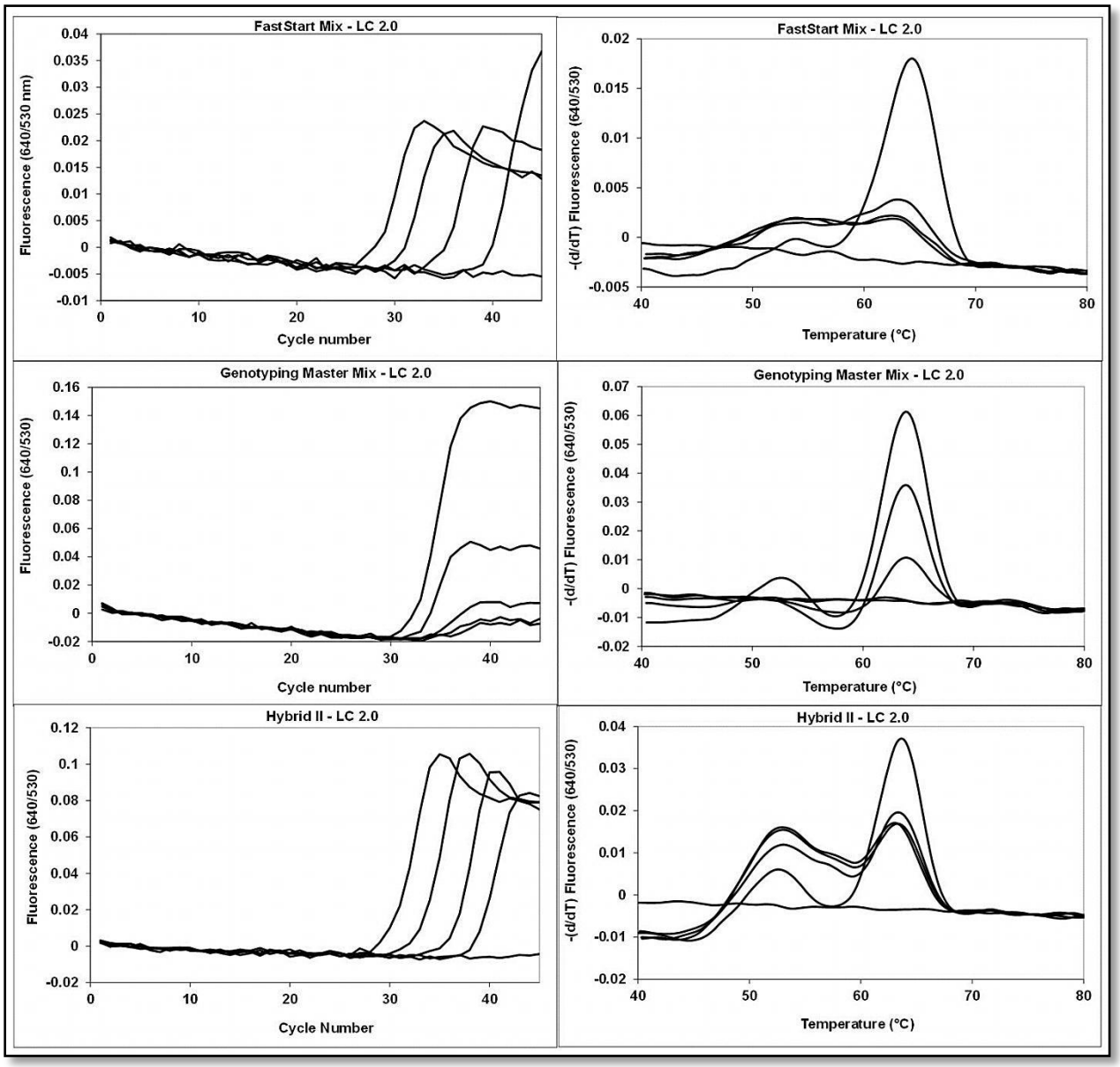


Figure 4.2: Effect of real-time PCR mixes on assay sensitivity and robustness using the LightCycler 2.0 instrument. Indicated are the amplification curves and melting profiles obtained using both the LightCycler® FastStart DNA MasterPLUS HybProbe or LightCycler® 480 Genotyping Master mixes, and a 1:1 mixture (Hybrid II). The *T. parva* gold standard positive control (KNP102) was serially diluted (10-fold). Crossing-point (CP) values were determined using the automated methodology implemented in the LightCycler software 4.0 for qualitative detection.

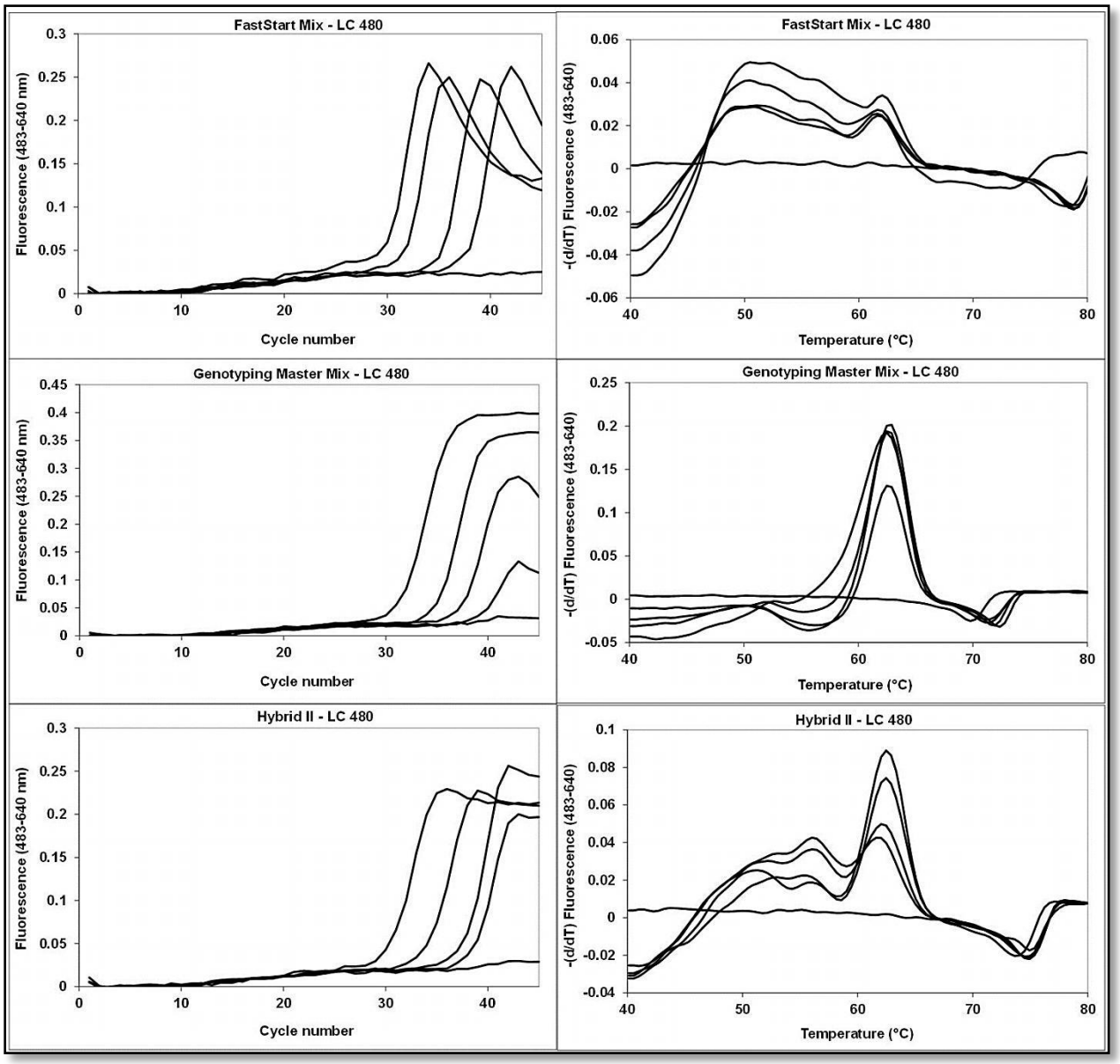


Figure 4.3: Effect of real-time PCR mixes on assay sensitivity and robustness using the LightCycler 480 instrument. Indicated are the amplification curves and melting profiles obtained using both the LightCycler® FastStart DNA MasterPLUS HybProbe or LightCycler® 480 Genotyping Master mixes, and a 1:1 mixture (Hybrid II). The *T. parva* gold standard positive control (KNP102) was serially diluted (10-fold). Crossing-point (CP) values were determined using the automated methodology implemented in the LightCycler software 4.0 for qualitative detection.

4.3.2 Specificity of the Hybrid II assay

At least nineteen different *Theileria* genotypes were identified in buffalo and cattle from southern Africa (Mans et al. 2011a). Samples that were *T. parva* negative on the real-time hybridization PCR (Sibeko et al. 2008), but positive for the other *Theileria* genotypes as well as other blood-borne parasites (babesias and trypanosomes) common to buffalo and/or cattle were tested using the Hybrid II assay (Fig. 4.4). No amplification or melting peaks were observed in any of the samples, except for *T. parva* and *T.*sp. (buffalo) (Fig. 4.4). Optimization of the PCR conditions using a touch-down protocol resulted in CP values consistently higher than 40 cycles observed for *T.*sp. (buffalo) positive samples. These samples also showed distinct melting peaks (57°C) that differ distinctly from that observed for *T. parva* (Fig. 4.4) and can as such be readily distinguished.

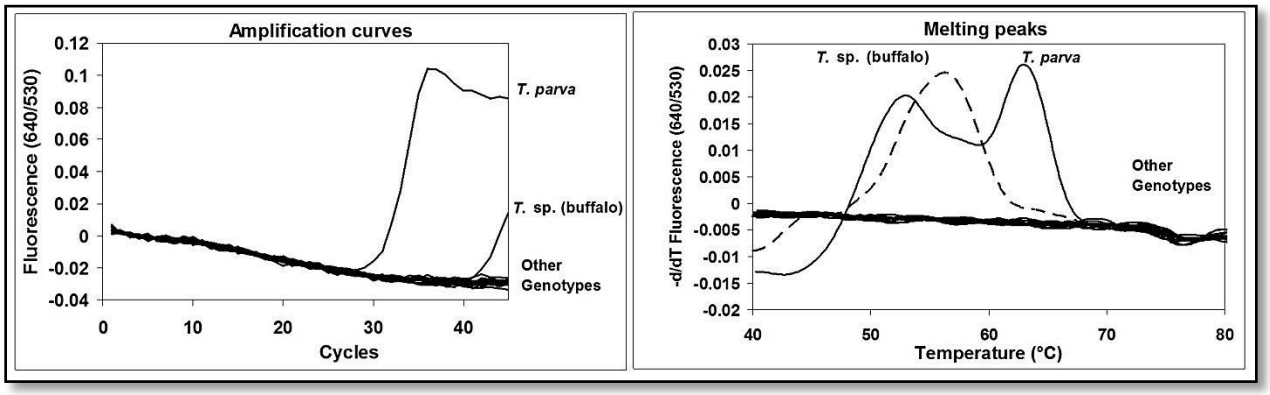


Figure 4.4: Specificity of the Hybrid II assay for various blood-borne parasites found in African buffalo and/or cattle. Amplification curves and melting peaks are indicated for *T. parva, T.* sp. (buffalo) and other genotypes that includes the *Theileria* species: *T. annulata*, *T. buffeli*-like C, *T. buffeli* type D-like, *T. mutans, T. mutans* like-1, *T. mutans* like-2, *T. mutans* like-3, *T. mutans* MSD, *T.* sp. (bougasvlei), *T.* sp. (duiker), *T.* sp. (kudu*)*, *T.* sp. (sable), *T.* sp. (sable-like), *T. taurotragi*, *T. velifera*, *T. velifera*-like A, *T. velifera*-like B, as well as the *Babesia* species: *B. bigemina* and *B. bovis* and the *Trypanosoma* species: *T. vivax*, *T. congolense S*avannah and *T. congolense* Kilifi.

4.3.3 Sensitivity of the Hybrid II assay

Using a defined template obtained from a quantified PCR product for *T. parva* (Pienaar et al. 2011a), it could be shown that the Hybrid II assay can detect up to ten copies of *T. parva* (Fig. 4.5A.) Using the gold standard control blood sample (buffalo KNP102) the Hybrid II detects a ten-fold serial dilution range to the same extent (up to $2x10^{-6}$% parasitemia), as the current hybridization test (Fig. 4.5B). Given this sensitivity range, a cut-off CP value for positive samples were determined below thirty-seven cycles. Crossing-point values higher than this should be considered false-positives, or should be investigated in more detail to confirm their *T. parva* positive status. This cut-off value also readily allows *T.*sp. (buffalo) positive samples to be identified.

4.3.4 Correlation of hybridization and Hybrid II assays for T. parva positive and negative samples

All samples that tested negative using the hybridization assay were negative on the Hybrid II assay, while 100% correlation was found for *T. parva* positive samples (Table 4.1). A linear correlation was found when CP values for 860 *T. parva* positive samples obtained with the two tests were compared (Fig. 4.6A). The majority (>95%) were found within 10% error deviation from the expected norm (Fig. 4.6A). Parasitemia ranges calculated for the buffalo samples ranged from 0.1 - 0.00001% and the frequency distribution were similar for both hybridization and Hybrid II assays (Fig. 4.6B).

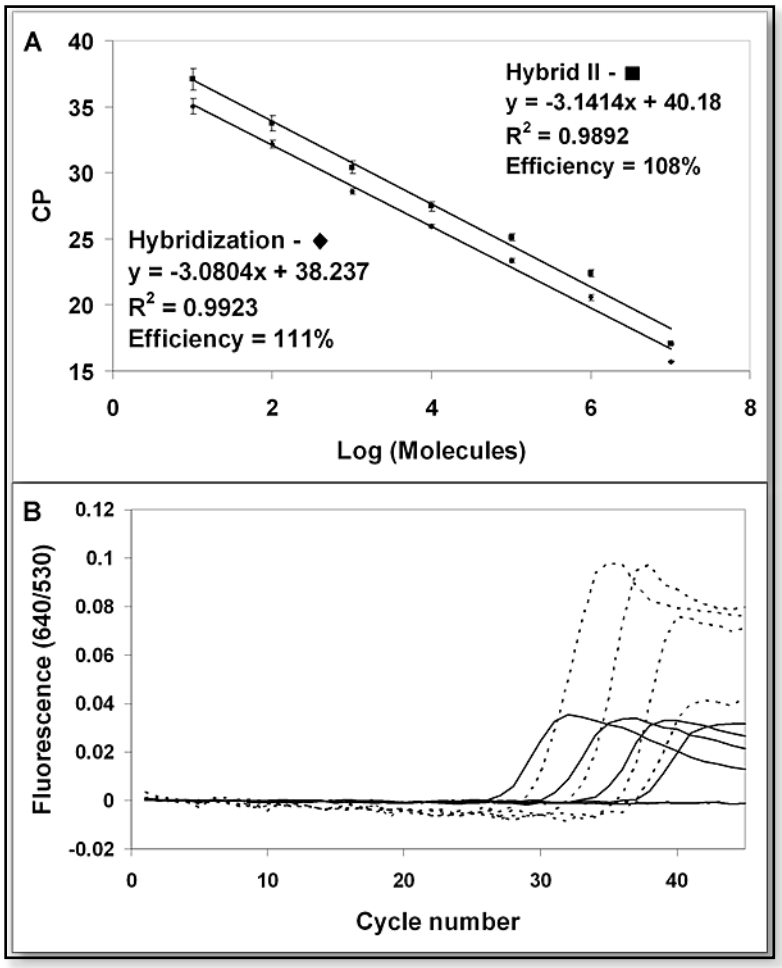


Figure 4.5: Analytical sensitivity of the Hybrid II assay. A) Comparison of the hybridization and Hybrid II assays using a *T. parva* template at defined concentrations related to number of molecules. Values indicated are in triplicate with standard deviation indicated by error bars. Linear regression lines were fitted through the points and the correlation is indicated. B) A ten-fold serial dilution series of the gold standard buffalo KNP102 (parasitemia ~$2E^{-3}$%-$2E^{-7}$%) was analyzed by the hybridization assay (solid lines) and Hybrid II assay (dotted line).

Table 4.1: Correlation of the Hybrid II assay and the hybridization assays. Sample status are indicated as previously determined using the hybridization assay and include negative (no amplification profiles or melting peaks at 640 or 705 nm), *T. parva* positive (amplification profiles and melting peaks at 640 and 705 nm) and *T.* sp. (buffalo)-like positive (no significant amplification profiles and melting peaks at 640 nm, high amplification and melting peaks at 705 nm).

Status	***Hybrid II***	**Hybridization**
Negative samples (n = 525)	525	525
***T. parva* positive samples (n = 860)**	860	860
T.* sp. (buffalo)-like positive samples (n = 983) that test negative for *T. parva	983	983
***T.* sp. (buffalo)-like positive samples that test negative for *T. parva* on the hybridization assay, but test positive on the Hybrid II assay**	53	N/A*

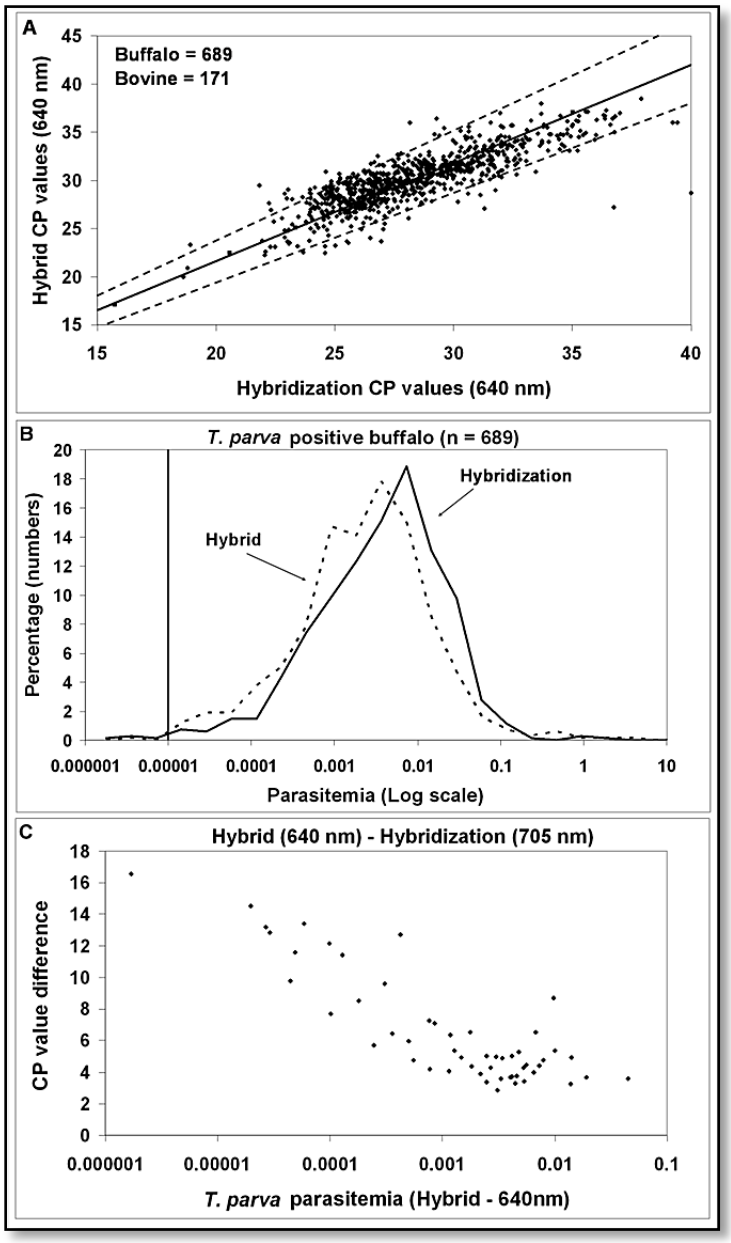


Figure4.6: Comparison between the Hybrid II and the hybridization assay. A) CP values from 860 *T. parva* positive samples are shown. Indicated is a linear regression line obtained for data from a defined PCR template for the Hybrid II and hybridization assay (Fig. 4.4A) (solid line) as well as 10% error deviation from this line (dotted lines). B) Parasitemias calculated for *T. parva* positive buffalo samples. Parasitemia was calculated from CP values obtained from the hybridization or Hybrid II assays using standard curves as previously described (Chapter 2; Pienaar et al., 2011a). The number of samples for each sample type analysed is indicated and the percentage of the total number was determined by grouping parasitemia's in 2-fold decreasing bins. The vertical line indicates the parasitemia at the cut-off point (CP value~37). C) The difference between CP values obtained with the Hybrid II (640nm) and the hybridization assay (705 nm) are indicated for samples considered to be *T. parva* negative with the hybridization assay. This is plotted against estimated *T. parva* parasitemia values obtained from the Hybrid II assay.

4.3.5 Effect of mixed-infections on T. parva detection

Samples that were *T.*sp. (buffalo)-like positive (n = 983) on the hybridization assay, tested negative with the Hybrid II assay (Table 4.1). However, 53 samples (~5%) considered to be *T. parva* negative on the hybridization assay, tested positive with the Hybrid II assay. The CP values for these samples fall well within the detection range of the Hybrid II assay with a mean CP value of 30.5±2.8. This corresponds to estimated parasitemia values of 0.00002-0.02%, which falls within the parasitemia ranges (0.0001 - 0.001) previously shown to be liable to *T.*sp. (buffalo) suppression (section 3.3.3) (Pienaar et al. 2011a). The majority of the CP values obtained for these samples on the hybridization assay at 705nm are lower compared to that of the Hybrid II assay, indicating that the *T.*sp. (buffalo)-like parasitemias are higher in these samples compared to that of *T. parva* (Fig. 4.6C).

The 53 samples that tested negative on the hybridization assay, but positive on the Hybrid II assay were further analysed by PCR using primers specific for different protein genes (p67N, p67C and p104). All 53 samples tested positive for *T. parva* using these genes (results not shown). In addition, when the *T.* sp. (buffalo) template concentration were suppressed by the presence of a locked nucleic acid (LNA) specific for *T.* sp. (buffalo), all tested positive in the hybridization assay (results not shown).

In order to confirm that the presence of *T.*sp. (buffalo) template does not affect the sensitivity of the Hybrid II assay, templates with defined ratios of *T.*sp. (buffalo): *T. parva* was used to simulate mixed infection parasitemias previously shown to affect the hybridization assay (section 3.2.3) (Pienaar et al., 2011a). No suppression was observed for the Hybrid II assay at ratios of *T.*sp. (buffalo): *T. parva* up to 1000:1 at *T. parva* parasitemia of 0.0001% (Fig. 4.7).

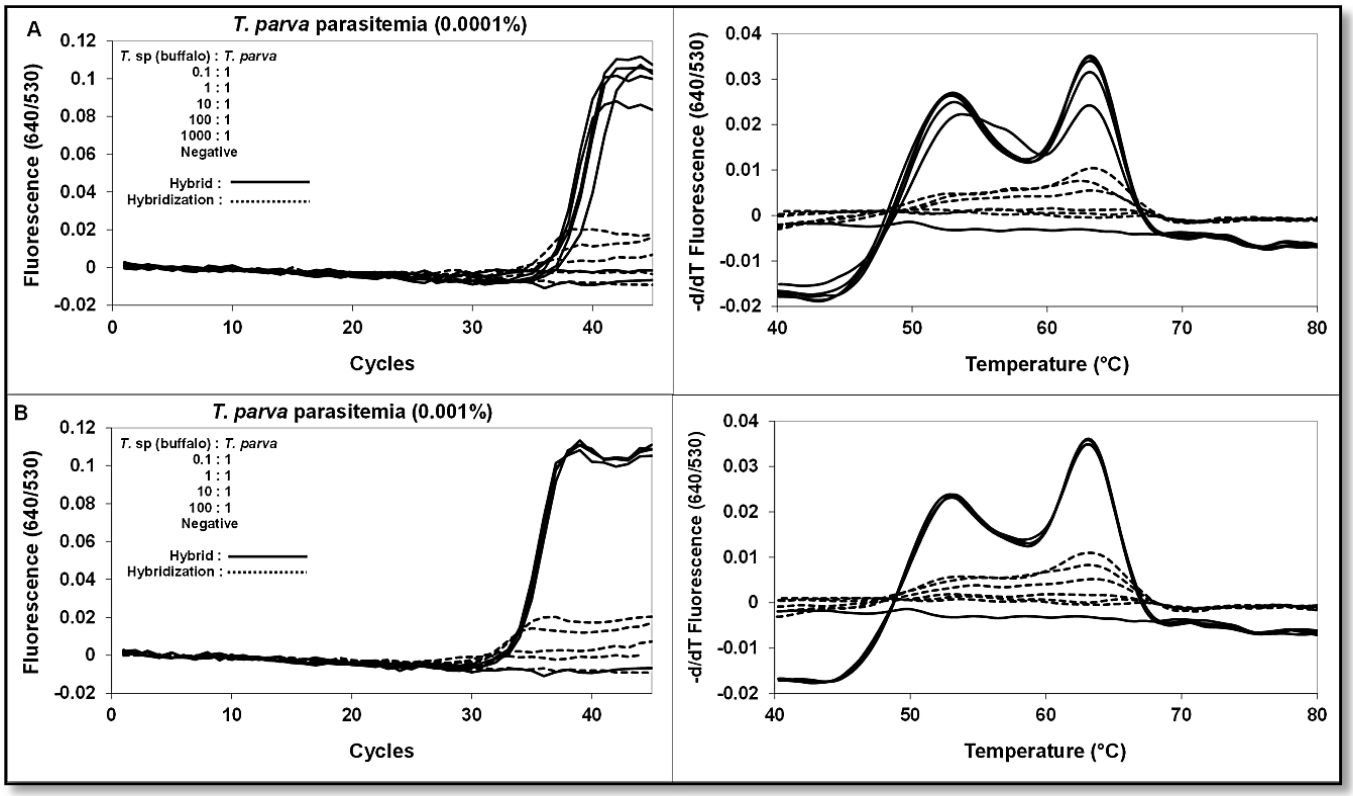


Figure4.7: The effect of mixed infections on the Hybrid II assay. DNA template that corresponds to *T. parva* parasitemia of A) 0.0001% and B) 0.001% were mixed with *T.* sp. (buffalo) DNA template at indicated ratios. Amplification curves and melting profiles are indicated for both Hybrid II (solid line) and hybridization assay (dotted line).

4.3.6 Detection of T. parva variants

A number of *T. parva* variant sequences were detected in buffalo and cattle samples (Fig. 4.1) (Mans et al. 2011a). The most prominent of these were variant 1 and variant 3 for which samples were identified that possessed these variants exclusively (Mans et al. 2011a). The Hybrid II assay detected these variants readily (Fig. 4.8). All samples in which variants were previously found (Mans et al. 2011a), tested positive for *T. parva* and the Hybrid II also detected cloned products (results not shown). *T.*sp. (buffalo) variants described (Mans et al. 2011a) did not give any signal (results not shown).

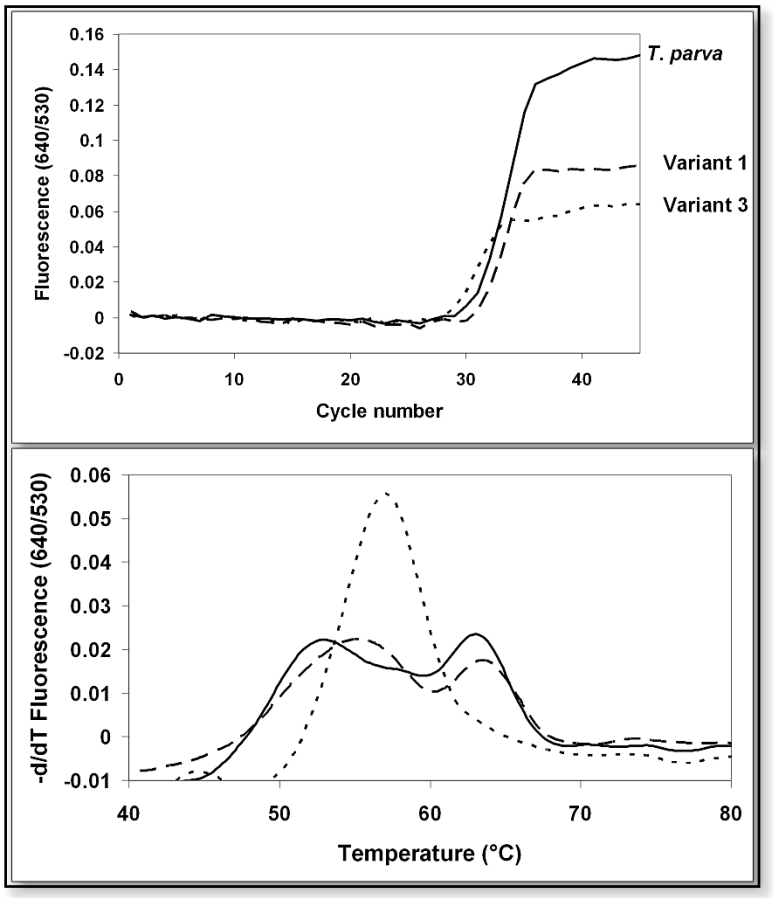


Figure 4.8: Detection of *T. parva* variants. Indicated are amplification curves and melting peaks for field samples that were positive for the *T. parva* variant 1 and variant 3 genotypes.

4.4 DISCUSSION

The accurate diagnosis of the carrier state of infectious agents in reservoir hosts is important to determine parasite prevalence within a host population. *Theileria* infections can be maintained for extended periods or even life long, during which time parasitemia can fluctuate significantly (Norval et al. 1992). The presence of multiple *Theileria* species is known to interfere with the accurate diagnosis of *T. parva*, using serological as well as nucleic acid based methods (Stoltsz, 1989; Pienaar et al. 2011a). The current Chapter describes the development of the Hybrid II assay, a sensitive real-time hybridization PCR test, for the detection of *T. parva* in carrier buffalo, which is not affected by mixed infections of *T. parva* and *T.*sp.(buffalo)-like parasites.

The Hybrid II assay combines reagent mixes used on the Roche LightCycler® 2.0 and LightCycler® 480 systems in order to obtain a mix that can be used on both instruments. This mix is more robust in regard to sensitivity, in analytical as well as qualitative terms than the individual mixes alone. The LightCycler® FastStart mix gives lower fluorescence signals for the melting peaks and are more prone to the hook effect (Barratt and MacKay, 2002), compared to the Hybrid II mix. A comparison of the real-time hybridization and Hybrid II assays shows that the Hybrid II mix was less prone towards the hook effect. During routine diagnostics using the hybridization assay it has been observed that the hook effect could severely suppress melting peak signals and could potentially lead to a false-negative diagnosis when melting peak analysis was used as criteria (personal observation). The Hybrid II mix could thus improve assays for both the LightCycler® 2.0 and LightCycler® 480 systems.

A number of blood-borne parasites are found in buffalo and/or cattle in southern Africa. These include *Babesia*, *Theileria* and *Trypanosoma* (Allsopp et al. 1999; de Vos and Potgieter, 1983; Mamabolo et al. 2009). The Hybrid II assay was specific for *T. parva* and did not detect representative samples from a variety of blood-borne parasites. The DNA templates for these parasites were all detectable by various forms of PCR and were as such considered to be present at levels that should be detectable by the Hybrid II assay. *T.*sp. (buffalo) positive samples did amplify after 40 cycles, however, it was determined that neither does this adversely affect the Hybrid II assay with regard to suppression of *T. parva* signal in the case of mixed infections, nor was it a source of false-positives (as in the case of the real-time hybridization PCR) due to the differences observed in melting peaks. Amplification of *T.*sp. (buffalo) is presumably due to mis-priming of the reverse primer. In the case of *T. parva* variants, any sample that shows CP values below the determined cut-off point (thirty-seven cycles) with aberrant melting peaks should be considered to be *T. parva* positive. It should be noted that for the current data set, less than 1% of all samples showed such variant profiles (personal observation). This was also observed with the real-time hybridization assay (Mans et al, 2011a). It is therefore not expected that variants will have a considerable effect on the diagnosis of *T. parva* using the Hybrid II assay. In cases where variant samples are identified, additional tests or cloning and sequencing can be performed to confirm *T. parva* status (Pienaar et al. 2011a).

The sensitivity of the Hybrid II assay was comparable to the hybridization PCR test (Sibeko et al. 2008). Despite the Hybrid II assay being approximately two-fold less sensitive than the latter test (CP values ~1-2 cycles higher), it was still within the dynamic range of the starting template tested (1-10 000 000 copies), performs as well and have the same detection limit as the hybridization PCR test using KNP102 serial dilutions.*T. parva* parasitemia ranges have been determined for Cape buffalo and were shown to range from 0.0001 - 0.1% for the majority (>98%) of samples (Pienaar et al., 2011a). This Chapter confirmed this using the Hybrid II assay and showed that frequency distribution profiles for *T. parva* parasitemia obtained for the Hybrid II and hybridization assays approximate normal distribution curves. This suggests that the parasitamias calculated represent actual parasitemia ranges found in buffalo. As such, the Hybrid II assay will also be useful to estimate parasitemia levels in infected carrier animals. The frequency distribution profiles indicate that a low number of infected buffalo (<1%) could potentially be missed using either the hybrid II or the hybridization assay due to low parasitemias. The use of a nested PCR, that is potentially more sensitive, was recently described for the p104 gene (Odongo et al. 2010). It should, however, be recognized that methods that rely on protein genes might not detect all sequence variants as the conservation of protein gene sequences is probably less than that of the 18S gene (Pienaar et al., 2011a). From a perspective of high-throughput routine diagnostics, nested PCR approaches are more time consuming and is as such, not practical. Other nucleic acid based assays that have been developed for the diagnosis of *T. parva* include the RLB assay (Gubbels et al. 1999), a Taqman hydrolysis probe assay based on the 18S gene (Papli et al. 2011) and LAMP assays based on the PIM and S5 ribosomal (annotated as the P150 gene) genes (Thekisoe et al. 2010). The Taqman and RLB, respectively, assays use universal *Theileria* and *Theileria/Babesia* primer sets. These assays have similar problems to that of the current hybridization PCR assay (Pienaar et al. 2011a; unpublished observation). The LAMP assays promise to be user-friendly, cost-effective and sensitive. In each case, three different primer sets binds within a ~200bp amplified region. Sequence variation within this relatively large region will severely affect the sensitivity of this assay.

It was previously shown that suppression of PCR signal can occur when mixed infections of *T. parva* and *T.*sp. (buffalo)-like parasites are encountered (Pienaar et al. 2011a). At low parasitemia levels (0.0001%) this can happen at ratios of *T.*sp. (buffalo): *T. parva* as low as 10:1,

while suppression at parasitemia levels of 0.001% were observed at ratios as low as 100:1 (Chapter 3; Pienaar et al. 2011a). The estimated *T. parva* parasitemias of the 53 samples that were not detected by the hybridization assay in the current study mostly fall within this range, while the *T.*sp. (buffalo)-like parasitemias is estimated to be at least 100-1000 fold higher. The fact that these samples tested positive in the presence of an LNA that suppress the DNA template concentration of *T.* sp. (buffalo), suggest that suppression of the PCR signal occurred in the hybridization assay. In contrast, not only did the Hybrid II assay detect these samples, but no suppression of signal occurred in mixed infection simulations of *T.* sp. (buffalo) and *T. parva* at ratios of 1000:1 at low *T. parva* (0.0001%) parasitemia levels. The Hybrid II assay therefore has the advantage, compared to the hybridization assay, that the presence of *T.*sp. (buffalo) will not affect the sensitivity of the test.

We found the Hybrid II assay to be an improvement on the current official real-time hybridization assay due to its robustness in the case of mixed infections of *T.*sp. (buffalo)-like parasites and *T. parva*. It was indicated in Chapter 3 that *T. parva* infections might be missed in ~10% of all mixed infections that occur in free ranging buffalo (Pienaar et al., 2011a). With regard to the diagnostic samples, this currently constitutes less than 0.5% of all samples and is not a problem of immediate concern (Pienaar et al., 2011a). As indicated, the number of *T.*sp. (buffalo)-like positive buffalo could increase in the future and have a more significant impact on diagnostics if *T. parva* is introduced into such herds (Pienaar et al. 2011a). Use of the Hybrid II assay will prevent this problem from occurring and presents a major advance in our ability to detect *T. parva* in Cape buffalo. This should improve the risk management of Corridor disease by the veterinary authorities.

Since the real-time hybridization PCR assay cannot distinguish between different *T.* sp. (buffalo)-like parasites, the question remain whether *T.* sp. (buffalo) or *T.* sp. (bougasvlei) is responsible for the majority of incidences of PCR suppression observed. This will be related to their parasitemia levels as well as geographic distribution. Their relationship to each other as well as *T. parva* also remains enigmatic. The next Chapter investigate these parameters.

CHAPTER 5

*Theileria sp. (buffalo) vs. Theileria sp. (bougasvlei)**

**(Work presented in this chapter was accepted for publication in Parasitology (Pienaar et al. 2014.)*

5.1 INTRODUCTION

The genus *Theileria* is part of the Piroplasmida (Phylum: Apicomplexa) and is transmitted by ixodid ticks to vertebrate hosts (Bishop et al. 2004). In the Bovini (African buffalo, Asiatic water buffalo and cattle) four main groups includes the *T. buffeli, T. mutans, T. velifera* and *T. taurotragi* clades (Chapter 1, Fig: 1.5) (Mans et al. 2011a). Genotypes within the *T. buffeli, T. mutans* and *T. velifera* clades are considered to represent populations of the same species (Chae et al. 1999; Gubbels et al. 2000). However, differences in host preference, clinical pathology, vector specificity and genetics are challenging this (Chae et al. 1999; Gubbels et al. 2000; Mans et al. 2011a; Mans et al. 2011b). Genotypes within these clades can have as many as 5-20 nucleotide differences in the 18S rRNA V4 hyper-variable region (Mans et al. 2011a). In contrast, differences in the "*T. taurotragi*" clade range from 3-15 nucleotides (Mans et al. 2011a). The *T. taurotragi* clade is composed of the recognized species *T. annulata, T. lestoquardi, T. parva* and *T. taurotragi* which have differentiated clinical outcomes in their vertebrate hosts and specific host and vector preferences (Bishop et al. 2004). The designation of the *T. buffeli, T. mutans* and *T. velifera* genotypes as representatives of the same species in their respective clades is linked to apathogenicity in the vertebrate host and the inability to differentiate them by non-genetic means (Chae et al. 1999). The question remains as to what level of genetic diversity would differentiate different *Theileria* species. In this regard, members of the "*T. taurotragi"* clade are of interest, since they are genetically more closely related than genotypes from other clades and may be good models for speciation in the *Theileria*.

Theileria sp. (buffalo) and *T.* sp. (bougasvlei) are from the "*T. taurotragi"* clade and related to *T. parva* (Zweygarth et al. 2009; Mans et al. 2011a; Pienaar et al. 2011a). Pairwise comparisons indicate 3-5 differences between these three genotypes in the 18S rRNA V4 hyper-variable region (Mans et al. 2011a). *Theileria* sp. (buffalo) was identified in African buffalo from East Africa based on serological differences with *T. parva* and later shown to be genetically distinct (Allsopp et al. 1993; Conrad et at. 1987). *Theileria* sp. (buffalo) and *T.* sp. (bougasvlei) was subsequently shown to be present in buffalo from southern Africa, to be apathogenic and has not yet been found in cattle (Allsopp et al. 1999; Oura et al. 2004; Oura et al. 2011; Mans et al. 2011a; Pienaar et al. 2011a). *Theileria* sp. (buffalo) and *T.* sp. (bougasvlei) affects accurate diagnostics of *T. parva* in African buffalo using the hybridization PCR assay due to PCR

suppression (Sibeko et al. 2008; Pienaar et al. 2011a). Accurate diagnostics of a *T. parva* carrier status in African buffalo is important in South Africa, since it is being used as a means to control Corridor disease outbreaks in cattle (Pienaar et al. 2011a).

The *T. parva* hybridization assay amplifies the 18S rRNA V4 hyper-variable region of *T. parva*, *T.* sp. (buffalo) and *T.* sp. (bougasvlei) (Sibeko et al. 2008; Pienaar et al. 2011a). Genotypes are distinguishable using two probes: the 640 nm probe detects *T. parva*, while the 705 nm probe detects the *Theileria* genus (Sibeko et al. 2008). The only non *T. parva* genotypes amplified by the primer set are *T.* sp. (buffalo) and *T.* sp. (bougasvlei) (Mans et al. 2011a). Since the 705 nm probe cannot differentiate these latter genotypes, they were collectively designated *T.* sp. (buffalo)-like parasites (Pienaar et al. 2011a). In cases where mixed infections of *T. parva* and *T.* sp. (buffalo)-like parasites occur with higher parasitemia for the latter parasites, false negative results for *T. parva* can be obtained (Pienaar et al. 2011a). It is not known which *T.* sp. (buffalo)-like genotype affects the hybridization assay to the largest extent. The geographical distribution of these parasites is unknown in southern Africa and it is unclear whether *T.* sp. (buffalo) and *T.* sp. (bougasvlei) belong to the same species or is closely-related species (Chaisi et al. 2011; Mans et al. 2011b). The current study aimed to address these questions by developing specific assays for *T.* sp. (buffalo) and *T.* sp. (bougasvlei), surveying a large geographically distinct *T.* sp. (buffalo)-like buffalo population as well as analysis of the COI gene.

5.2 MATERIALS AND METHODS

5.2.1 Buffalo samples and DNA extraction

African buffalo blood samples from game ranches and National Parks in southern Africa were submitted to the Parasites, Vectors and Vector-Borne Diseases laboratory during 2008-2011 for *T. parva* diagnostics using the real-time hybridization assay (Sibeko et al., 2008). Genomic DNA was extracted from 200 µl of whole blood using the MagNa Pure Large Volume Kit and MagNa Pure LC (Roche Diagnostics). DNA was eluted in 100 µl of elution buffer and 2.5 µl (~15-50 ng/µl DNA) used per real-time hybridization assay (Pienaar et al., 2011a). For all assays the gold standard *T. parva* positive (KNP102) (Sibeko et al., 2008), *T.* sp. (buffalo) positive (Buffalo 114) (Zweygarth et al. 2009), and negative (9426) controls used for routine

diagnostics were included. The negative control was born and raised in a herd under quarantined tick-free conditions. The*T.* sp. (bougasvlei) samples used were negative for *T.* sp. (buffalo) and *T. parva* by reverse line blot (RLB) (Pienaar et al. 2011a) as well as cloning and sequencing of the 18S rRNA gene (Mans et al. 2011a).

5.2.2 Real-time hybridization assay for T. parva

The *T. parva* real-time hybridization assay was performed (Pienaar et al. 2011a), using the LightCycler® 2.0 (Roche Diagnostics, Mannheim, Germany). Briefly, assay conditions included 4 µl of the LightCycler-FastStart DNA MasterPlus Hybridization mix (Roche Diagnostics, Mannheim, Germany), 1U uracil deoxy-glycosylase (UDG) (Roche Diagnostics, Mannheim, Germany), 0.5 pmol TpF forward and TgR reverse primers, 0.1 pmol of the *T. parva* specific anchor and probe, 0.1 pmol of the *Theileria* genus specific anchor and probe pairs at a final volume of 20 µl. Crossing-point (CP) values were calculated by the qualitative analysis mode of the LightCycler 4.0 software (Roche Diagnostics, Mannheim, Germany). Results were interpreted according to the protocol: negative samples show no amplification or melting curves for the 640 or 705 nm channels. *Theileria parva* positive samples show amplification and melting curves in both 640 and 705 nm channels. Weak amplification and melting curves for the 640 nm channel, but significant signals for the 705 nm channel, indicate samples as *T. parva* negative, but *T.* sp.(buffalo)-like positive (Pienaar et al. 2011a).

5.2.3 Hybrid II assay for T. parva

The Hybrid II assay was performed (Pienaar et al. 2011b) using 2 µl LightCycler-FastStart DNA MasterPlus Hybridization mix (Roche Diagnostics, Mannheim, Germany), 2 µl LightCycler® 480 Genotyping Master mix (Roche Diagnostics, Mannheim, Germany), 1 U uracil deoxy-glycosylase (UDG) (Roche Diagnostics, Mannheim, Germany), 0.5 pmol TgF forward and TpR reverse primer, 0.1 pmol each of the *T. parva* specific anchor and probe pairs (final volume of 20 ul). Reaction conditions included an initial UDG activation step (40°C, 10 min) and a pre-incubation step (95°C, 10 min). An initial 10 cycles of denaturation (95°C, 10s), annealing (60°C, 10s) and extension (72°C, 15s), followed by a touch-down procedure (60-56°C,

15 cycles), followed by 20 cycles at 56°C. Melting curves were obtained using a ramp rate of 0.2°/s from 40-95°C. These conditions were used on both Roche LightCycler® 2.0 and LightCycler® 480 systems (Roche Diagnostics, Mannheim, Germany).

5.2.4 Real-time hybridization assay for T. sp. (buffalo) and T. sp. (bougasvlei)

The real-time hybridization assays for *T.* sp. (buffalo) and *T.* sp. (bougasvlei) utilized the same primer set and 640 nm anchor probe as the *T. parva* hybridization assay. However, unique sensor probes were used for *T.* sp. (buffalo) (LC640-TC**A**gACggAgTT**TA**CT-PH) and *T.* sp. (bougasvlei) (LC640-TC**A**gACg**A**AgTT**TC**TT-PH), where the bases in bold indicate locked nucleic acids. Assay conditions included 2 µl LightCycler-FastStart DNA MasterPlus Hybridization mix (Roche Diagnostics, Mannheim, Germany), 2 µl LightCycler® 480 Genotyping Master mix (Roche Diagnostics, Mannheim, Germany), 1 U uracil deoxy-glycosylase (UDG) (Roche Diagnostics, Mannheim, Germany), 0.5 pmol forward and reverse primer, 0.1 pmol each of the hybridization anchor and probe pairs (final volume of 20 ul). Reaction conditions included UDG activation (40°C, 10 min) and a pre-incubation (95°C, 10 min), followed by 45 cycles of denaturation (95°C, 10s), annealing (58°C, 10s) and extension (72°C, 15s). Melting curves were obtained by ramping from 40°C-75°C (5 data acquisitions per degree). Runs were performed using the LightCycler® 480 system and CP values calculated using the LightCycler Version 1.5.0 software.

5.2.5 Specificity of T. sp. (buffalo) and T. sp. (bougasvlei) hybridization assays

Samples positive for *T. parva*, *T.* sp. (buffalo) and *T.* sp. (bougasvlei) were from the positive control panel. Buffalo or cattle samples negative with the hybridization assay for *T. parva* or *T.* sp. (buffalo)-like parasites were analyzed by RLB (Pienaar et al., 2011a) and identified samples positive for *Babesia bigemina* and *B. bovis,Anaplasma centrale* and *A. marginale*, *Ehrlichia ruminantium*, *Theileria annulata*, *T. lestoquardi*, *T.* sp. (duiker), *T.* sp. (kudu*)* and *T.* sp. (sable). The 18S gene for various *Theileria* species was also amplified, cloned and sequenced (Mans et al., 2011a) and this identified samples positive for *T. buffeli*-like C, *T. buffeli* type D-like, *T. mutans*, *T. mutans* like-1, *T. mutans* like-2, *T. mutans* like-3, *T. mutans* MSD, *T.* sp. (sable-like),

T. taurotragi, *T. velifera*, *T. velifera*-like A and *T. velifera*-like B. Cattle samples positive for the *Trypanosoma* spp. *T. vivax*, *T. congolense* Savannah and *T. congolense* Kilifi were confirmed by cloning and sequencing of the 18S rRNA gene (Mamabolo et al., 2009). Samples from the KNP were also analyzed using the RLB assay to detect infection with *Theileria* parasites (Pienaar et al. 2011a).

5.2.6 Estimation of parasitemia in database samples

Parasitemias for *T. parva* positive samples were estimated as described (Pienaar et al. 2011a). For *T.* sp. (buffalo) and *T.* sp. (bougasvlei), standard curves for the real-time hybridization assays were constructed using 18S rRNA PCR templates quantified by nanodrop spectrophotometry and gel electrophoresis against DNA standards. These concentrations were converted to molecules and estimated parasitemia (Pienaar et al. 2011a) (see calculations hereafter). Ten-fold serial dilutions were made to span CP values of ~15-40 cycles, corresponding with the observed ranges of field and diagnostic samples. From regression curves, parasitemias were calculated from CP values. In each run, a positive control for *T. parva*, *T.* sp. (buffalo) or *T.* sp. (bougasvlei) was included to estimate the consistency of the real-time hybridization assay. Mean CP values for the KNP102 samples during the assays were 27.73±0.55 (n=220), for *T.* sp. (buffalo) it was 33.26 ±0.47 (n=15) and for *T.* sp. (bougavslei) it was 28.93±0.65 (n=15).

Sensitivity calculations for of *T.* sp. (buffalo) and *T.* sp. (bougasvlei) real-time assays:

***T.*sp. (buffalo)**

Have ~10 ng/ul cleaned up product

A 1101bp fragment is amplified with Allsopp primers	1101
Ave nucleotide =	615 (or g/mole)
Mass of 1101 bp fragment =	677115 g/mole
No of moles in 10 ng of 1101 bp =	1.47685×10^{-14} moles
No of molecules/mole =	8.89×10^{9} molecules/mole
BUT have 2 18S copies in 2.5 µl used/reaction	
Thus no. of molecules/µl =	2.22×10^{10} molecules/µl
This is equal to	1.11×10^{10} genomic equivalents

Serial dilution	**Genomic equivalents**	**Per 2.5µl PCR reaction**	**Dilution factor**	**ng/µl**	**Cp**
3x diluted from 46 ng/µl Neat	1.11×10^{9}	2.22×10^{9}		15.33333333	5
1	1.11×10^{8}	2.22×10^{8}	= 10X	10	10.23
2	1.11×10^{7}	2.22×10^{7}	= 1X	1	13.77
3	1.11×10^{6}	2.22×10^{6}	9	0.1	17.03
4	1.11×10^{5}	2.22×10^{5}	8	0.01	20.91
5	1.11×10^{4}	2.22×10^{4}	7	0.001	23.99
6	1.11×10^{3}	2.22×10^{3}	6	0.0001	27.66
7	1.11×10^{2}	2.22×10^{2}	5	0.00001	30.93
8	1.11×10^{1}	2.22×10^{1}	4	0.000001	34.31
9	1.11	2.22	3	0.0000001	37.68
10	1.11×10^{-1}	2.22×10^{-1}	2	0.00000001	
11	1.11×10^{-2}	2.22×10^{-2}	1	0.000000001	

Sensitivity calculations for of *T.* sp. (buffalo) and *T.* sp. (bougasvlei) real-time assays:

***T.*sp. (bougasvlei)**

Have ~10 ng/ul 1101 bp 18S rRNA product
A 1101 bp fragment is amplified with Allsopp et al. (1993)primers.

Ave nucleotide =	615 (or g/mole)
Mass of 1101 bp fragment =	677115 g/mole
No of moles in 10 ng of 1101 bp =	1.47685×10^{14} moles
No of molecules/mole =	8.89×10^{9} molecules
BUT have 2 18S copies in 2.5 ul used/reaction	
Thus no. of molecules/µl =	2.22×10^{10} molecules/µl
This is equal to	1.11×10^{10} genomic equivalents

Serial dilution 3x dilution of 50 ng/µl Neat	**Genomic equivalents**	**Per 2.5ul PCR reaction**	**Dilution factor**	**ng/ul**	**Cp**
				16.66666667	9.98
Y41-7/1	1.11×10^{10}	2.2×10^{10}	= 10X	10	11.27
Y41-7/2	1.11×10^{9}	2.2×10^{9}	= 1X	1	14.67
Y41-7/3	1.11×10^{8}	2.2×10^{8}	9	0.1	17.75
Y41-7/4	1.11×10^{7}	2.2×10^{7}	8	0.01	20.95
Y41-7/5	1.11×10^{6}	2.2×10^{6}	7	0.001	24.98
Y41-7/6	1.11×10^{5}	2.2×10^{5}	6	0.0001	28.02
Y41-7/7	1.11×10^{4}	2.2×10^{4}	5	0.00001	31.06
Y41-7/8	1.11×10^{3}	2.2×10^{3}	4	0.000001	34.1
Y41-7/9	1.11×10^{2}	2.2×10^{2}	3	0.0000001	37.14
	1.11×10^{1}	2.2×10^{1}	2	0.00000001	
	1.111701	2.22340	1	0.000000001	

5.2.7 Calculation of the coefficient of correlation parameter Rij

The coefficient of correlation (*Rij*) was calculated as described to investigate competitive exclusion between parasites (Dib et al. 2008) using Microsoft Excel. Briefly, the presence or absence of a parasite in a given sample is treated as a phenotypic character with absence represented by a value of 1 and presence represented by a value of 2. The coefficient of correlation (*Rij*) represent the association between a pair of parasites designated *i* and *j*. It is calculated using the formula:

$$Rij = \frac{\frac{n}{n-1}\left[\frac{n11}{n} - \frac{(n11+n12)}{n}\frac{(n11+n21)}{n}\right]}{\sqrt{\frac{(n11+n12)}{n}\frac{(n22+n21)}{n}\frac{(n11+n21)}{n}\frac{(n22+n12)}{n}}}.$$

Values denote the following: n: total sample number tested; n11: samples negative for both parasites *i* and *j*; n12: samples negative for parasite *i*, positive for *j*, n21: samples positive for parasite *i*, negative for *j* and n22: samples positive for both parasites *i* and *j*. Due to character coding and the oriented nature of the formula, a positive value for *Rij* imply positive correlation between parasite pairs and a negative value imply avoidance or competitive exclusion between parasites.

5.2.8 Cloning and sequencing of the COI gene

Genetic differentiation was observed between *T.* sp. (buffalo) and *T.* sp. (bougasvlei) using the S5 nuclear ribosomal gene (Mans et al. 2011a). To find genetic markers unbiased with regard to potential linkage, the mitochondrial COI gene was investigated. Samples positive for *T. parva*, *T.* sp. (buffalo) and *T.* sp. (bougasvlei) were selected from different geographic areas. Samples positive for *T. taurotragi* and *T. lestoquardi* were also analyzed as well as an unknown genotype. *Theileria* genotypes were amplified using the COI primer sets (ThCOIF1/ThCOIR1 and ThCOIF2/ThCOIR2), used for genetic barcoding of the Piroplasmida (Gou et al. 2012). These primers did not work for the amplification of *T. parva* and *T.* sp. (buffalo), while a primer set (ThCOIF1/ThCOIR1) that did work for *T.* sp. (bougasvlei) also amplified another *Theileria* species.

New primer sets were designed specific for *T. parva,T.* sp. (buffalo), *T. lestoquardi* and *T. taurotragi* (COIF: ACT GGT CTT TTT GGA GGA; COIR: TCT GGT ATT CTT CTT GGA A) and for *T.* sp. (bougasvlei) (BgvlF: GTA TGA GTG GAT TAA AAG TGA; BgvlR: TTC TTC TTG GTA AAG GTG AG). Assay conditions for PCR consisted of 25 µl GreenTaq (Fermentas), 21.5 µl water, 1 µl primer mix (10 pmol forward and reverse primer, respectively) and 2.5 µl sample. Reaction conditions included denaturation (94°C, 2 minutes), 45 cycles of denaturation (94°C, 30seconds), annealing (53°C, 30seconds) and extension (72°C, 2 minutes), with a final extension (72°C, 7 minutes). Samples were separated on a 1.2% agarose gel using standard TAE

buffer and visualized using Ethidium Bromide. Bands were cut from the gel, purified using the Silica Bead DNA Gel Extraction Kit (Thermo Scientific) and eluted with 15 µl of water. Purified bands were A-tagged by mixing equal volumes of sample and Greentaq (10 µl), denaturing at 94°C (2 minutes) and extension at 72°C (7 minutes). Products were cloned into the pGem T-Easy vector (Promega), transformed into competent *E. coli* cells and colonies screened using gene specific primers. For each sample, three positive clones were purified using silica beads and sequenced from both directions using gene specific primers and the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Consensus sequences were determined for the clones.

5.2.9 Bioinformatic analysis of the COI gene

Sequences were aligned with ClustalX (Jeanmougin et al. 1998), manually checked and trimmed to an open reading frame (777 bp, 259 amino acids). Bayesian analysis was performed using MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003). Codons were partitioned into three sets corresponding to the first, second and third positions. Partitions were allowed to have different rates and a general time reversible (GTR) model of nucleotide substitution were used with a proportion of invariant sites and a gamma distribution of among site heterogeneity using the nst = 6 rates = ingamma command. Four categories were used to approximate the gamma distribution and two runs were performed simultaneously, each with four Markov chains (one cold, three heated) which ran for 4,000,000 generations. The first 2,000,000 generations were discarded (burnin) and every 100th tree sampled to calculate a 50% majority-rule consensus tree. Nodal values represent posterior probability that the recovered clades exist given the sequence dataset and are considered significant above 95% (Alfaro et al., 2003).

Pairwise genetic distances were calculated from the alignment using the Tamura-Nei (1993) model in Mega 5 (Tamura et al. 2011). Rate variation among sites was modeled with a gamma distribution (shape parameter = 0.41) and differences in the composition bias among sequences were considered in evolutionary comparisons (Tamura and Kumar, 2002). The analysis involved 97 nucleotide sequences and included all codon positions. Positions containing gaps and missing data were eliminated with 775 positions in the final dataset.

The alignment was submitted to the Automatic Barcode Gap Discovery (ABGD) Server (http://wwwabi.snv.jussieu.fr/public/abgd/) that calculates a barcode gap from the inference of a model-based one-sided confidence limit for intraspecific divergence (Puillandre et al. 2011). Minimum prior intraspecific divergence (0.001) and maximum prior intraspecific divergence (0.1) were scanned over 10 steps with a minimum gap width (1.5) and distance distribution Nn bins (20). Pairwise-distances were calculated using the Kimura two parameter model (K2P). Iterative limit inference and gap detection results in partitioning of data into groups predicted to be species units.

5.3 RESULTS

5.3.1 Specificity and sensitivity of the T. sp. (buffalo) and T. sp. (bougasvlei) hybridization assays

Each assay showed specific detection of their respective genotypes at 640 nm (Fig. 5.1). In addition, no other *Theileria* genotype thus far detected in African buffalo gave any amplification signal based on the 640 nm probes (Fig. 5.1). Both assays detected ten copies of template DNA with efficiencies of 92-95% (Fig. 5.2). Given this sensitivity range, a cut-off CP value for positive samples were determined to be below thirty-seven cycles for both assays.

Samples were analyzed using the *T. parva* hybridization assay (n=1301). Of these, 1211 were positive at 705 nm, indicating both *T. parva* and *T.* sp. (buffalo)-like infections (Table 5.1).

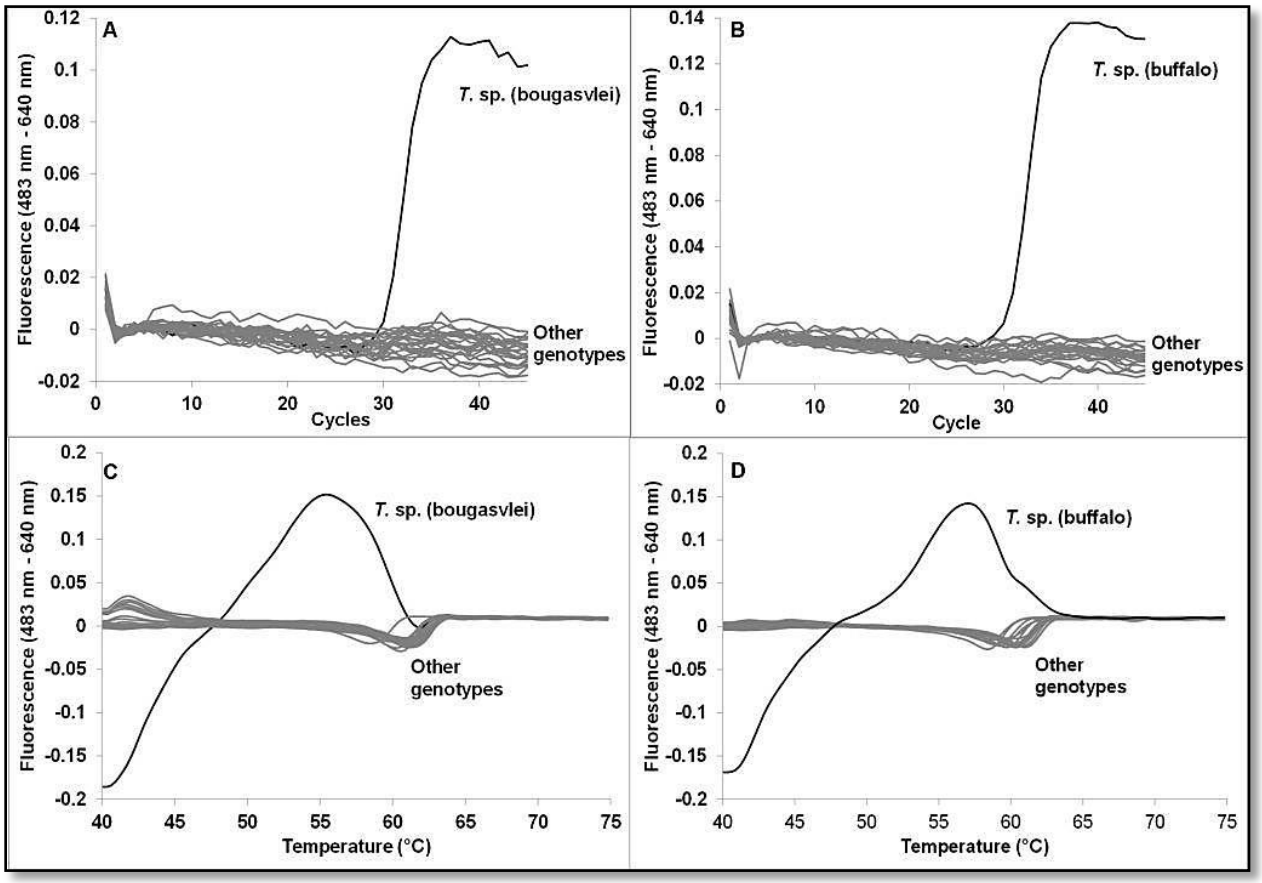


Figure 5.1: Specificity of the *T.* sp. (buffalo) and *T.* sp. (bougasvlei) real-time hybridization PCR assays. Indicated are amplification curves (A, B) and melting curves (C, D) for the *T.* sp. (buffalo) and the *T.* sp. (bougasvlei) positive controls. Other genotypes refer to negative control and samples that possessed genotypes found in bovids. *Theileria* species included: *T.* sp. (bougasvlei) and *T.* sp. (buffalo), respectively, *T. annulata*, *T. buffeli*-like C, *T. buffeli* type D-like, *T. lestoquardi*, *T. mutans*, *T. mutans* like-1, *T. mutans* like-2, *T. mutans* like-3, *T. mutans* MSD, *T. parva*, *T.* sp. (duiker), *T.* sp. (kudu), *T.* sp. (sable), *T.* sp. (sable-like), *T. taurotragi*, *T. velifera*, *T. velifera*-like A, *T. velifera*-like B. *Babesia*: *B. bigemina* and *B. bovis*. *Anaplasma*: *A. centrale* and *A. marginale*. *Ehrlichia:E. ruminantium*. *Trypanosoma* species: *T. vivax*, *T. congolense* Savannah and *T. congolense* Kilifi.

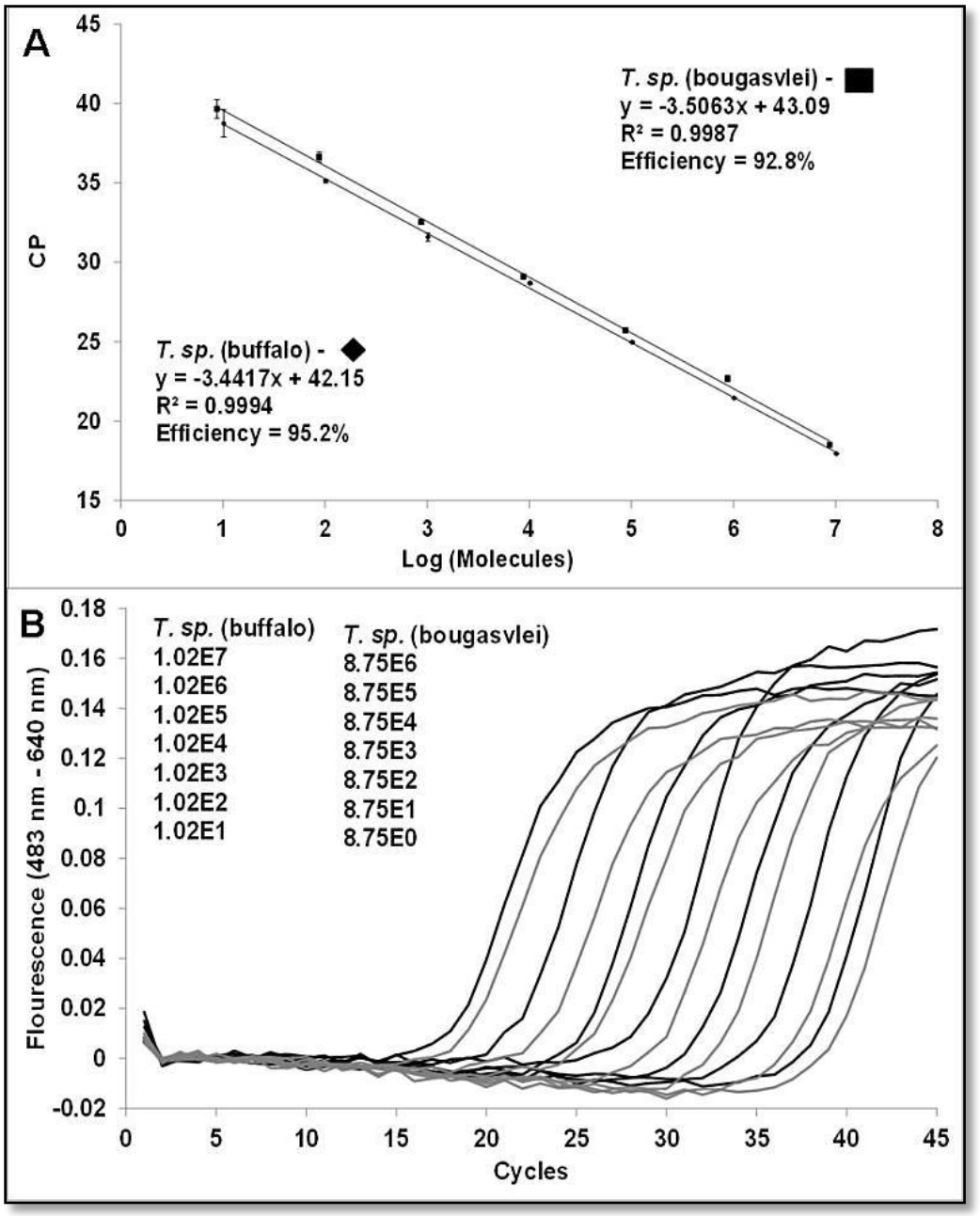


Figure 5.2: Analytical sensitivity of the *T.* sp. (buffalo) and *T.* sp. (bougasvlei) assays. A) Standard curves obtained from a 10-fold serial dilution of *T.* sp. (buffalo) and *T.* sp. (bougasvlei) 18S DNA templates. The number of molecules vs their corresponding CP values is indicated. B) Amplification curves of the serial dilution indicating *T.* sp. (buffalo) (black curves) and *T.* sp. (bougasvlei) (grey curves).

Table 5.1: Diagnostic results for *T. parva* (Tpar), *T.* sp. (buffalo) (TsB) and *T.* sp. (bougasvlei) (Bgvl).

Locality	Tpar	TsB	Bgvl	Tpar / TsB	Tpar / Bgvl	Tpar / TsB / Bgvl	TsB / Bgvl	705 Neg	Total
Kruger National Park (KNP)	16	3	15	101	63	27	1	39	265
Hluhluwe Game Reserve (HGR)	0	8	0	91	0	0	0	0	99
Chobe National Park (CNP - Botswana)	1	0	2	0	27	0	0	0	30
Marakele National Park (MNP)	0	2	0	42	0	0	0	0	44
Hwange National Park (HNP - Zimbabwe)	2	0	0	0	21	2	0	0	25
Gonarezhou National Park (GNP – Zimbabwe)	0	0	0	0	9	1	0	0	10
Great Limpopo Trans frontier park (GLTP – Sengwe corridor, Zimbabwe)	0	0	0	7	1	7	0	0	15
Niassa National Reserve (NNR - Mozambique)	2	3	2	15	0	3	2	0	27
Great Limpopo Trans frontier park (GLTP – Manguana Powerline, Mozambique)	0	2	2	12	2	8	1	0	27
Khaudum Game Reserve (KGR - Namibia)	0	0	24	0	0	0	0	0	24
Diagnostic	170	318	107	63	19	0	7	51	735
Total	191	336	152	331	142	48	11	90	1301
Percentage	14.7	25.8	11.7	25.4	10.9	3.7	0.8	6.9	100

5.3.2 Sample analysis

All 705 nm positive samples were tested using the hybrid II assay for *T. parva*, and the *T.* sp. (buffalo) and *T.* sp. (bougasvlei) assays. Samples were positive for *T. parva* (n=712), *T.* sp.

(buffalo) (n=726) and *T.* sp. (bougasvlei) (n=353). Animals with single infections included for *T. parva* (n=191), *T.* sp. (buffalo) (n=336) and *T.* sp. (bougasvlei) (n=152). Mixed infections of *T. parva* and *T.* sp. (buffalo) or *T.* sp. (bougasvlei) comprised 331 and 142 animals, respectively. In contrast, animals with mixed infections of *T.* sp. (buffalo) and *T.* sp. (bougasvlei) comprised only 59 animals, of which 48 were also infected with *T. parva*. In addition 90 samples negative at 705 nm were tested that was negative using all different tests.

5.3.3 Geographic distribution

Theileria sp. (buffalo) and *T.* sp. (bougasvlei) were distributed across southern Africa, although the majority of buffalo ranches and game parks possessed a single genotype (Fig. 5.3).The majority of mixed infections of *T.* sp. (buffalo) and *T.* sp. (bougasvlei) were limited to NationalParks, while seven diagnostic samples were limited to two localities. Even though both genotypes occur in the Kruger National Park (KNP), their predominant distribution was limited to specific geographic areas (Fig. 5.4A). *Theileria* sp. (buffalo) occurred in the northern and southern areas of the park, with *T.* sp. (bougasvlei) in the central region. Animals that possessed mixed-infections occurred at *T.* sp. (buffalo) and *T.* sp. (bougasvlei) interfaces. To determine whether the disparate distribution of *T.* sp. (buffalo) and *T.* sp. (bougasvlei) is not an artifact, co-infection rates with other *Theileria* species were determined for the KNP dataset (Fig. 5.4B). Co-infection of *T.* sp. (buffalo) and *T.* sp. (bougasvlei) was lower than any other parasite pair, while co-infection of either *T.* sp. (buffalo) or *T.* sp. (bougasvlei) with other *Theileria* species was comparable.

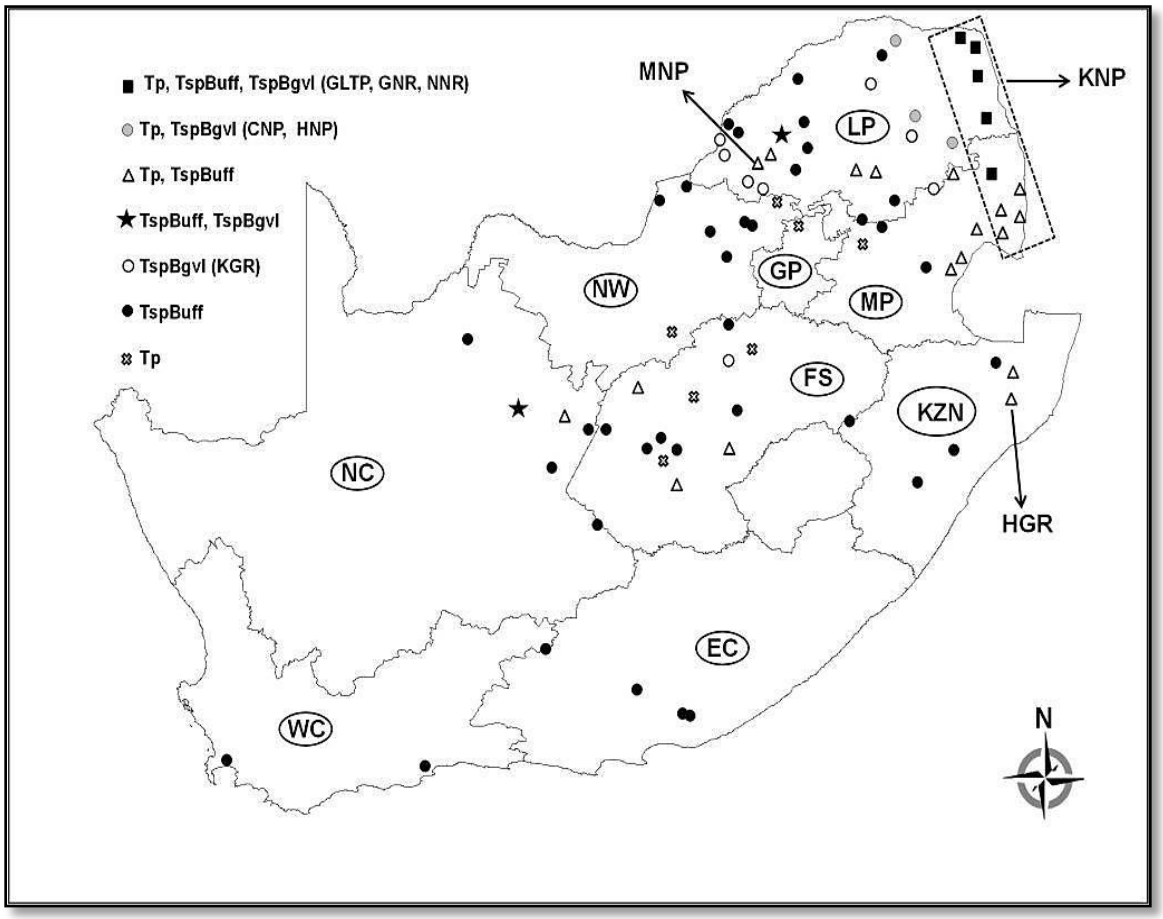


Figure 5.3: Geographic distribution of *T. parva*, *T.* sp. (buffalo) and *T*. sp. (bougasvlei) in South Africa. National parks are indicated with arrows (KNP: Kruger National Park; HGR: Hluhluwe Game Reserve; MNP: Marakele National Park). All other localities include small game reserves, commercial wild life ranches and buffalo project ranches grouped under diagnostic samples. Distribution in international parks is indicated in parenthesis (CNP: Chobe National Park; GLTP: Great Limpopo Transfrontier Park; GNP: Gonarezhou National Park; HNP: Hwange National Park; KGR: Khaudum Game Reserve; NNR: Niassa National Reserve). Circles indicate provinces: Western Cape (WC), Northern Cape (NC), Eastern Cape (EC), North-West (NW), Free State (FS), Gauteng (GP), Kwa-Zulu Natal (KZN), Mpumalanga (MP) and Limpopo (LP).

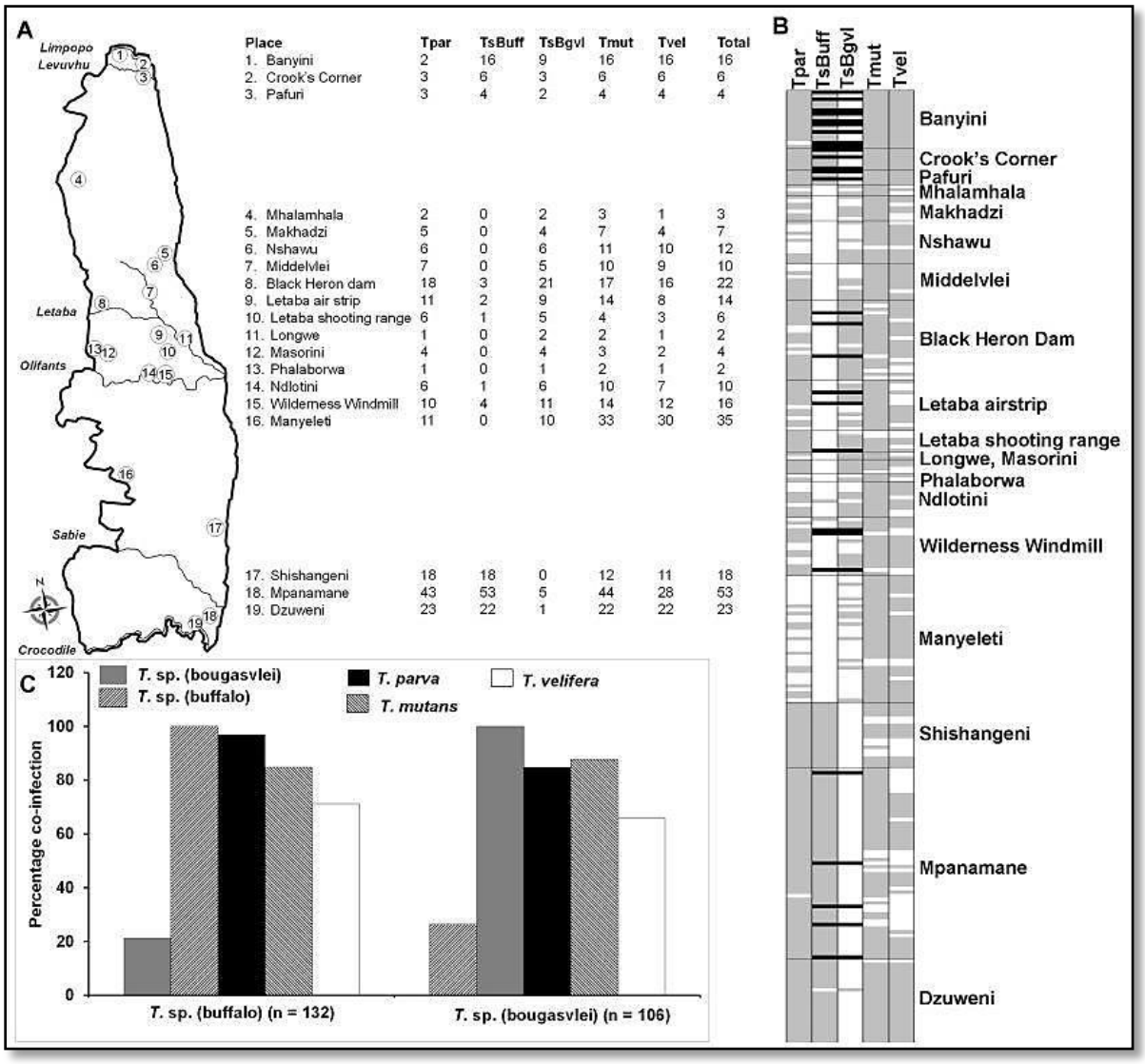


Figure 5.4: Distribution of *T. parva*, *T.* sp. (buffalo) and *T.* sp. (bougasvlei) in the Kruger National Park. A) Park boundaries are indicated with bold lines and major rivers by thin lines and italicized names. Sampling sites are indicated with numbered circles and corresponding names and the number of positive samples per site found for *T. parva* (Tpar), *T.* sp. (buffalo) (TsBuff), *T.* sp. (bougasvlei) (TsBgvl), *T. mutans* (Tmut) and *T. velifera* (Tvel). B). A heat map distribution indicates absence (white), presence (grey) or mixed-infections for *T.* sp. (buffalo) and *T.* sp. (bougasvlei) (black). C) Percentage co-infection for *T.* sp. (buffalo) or *T.* sp. (bougasvlei) positive buffalo with *T. parva*, *T. mutans* and *T. velifera*. The presence of the latter two parasites was determined using RLB analysis.

5.3.4 Parasitemia levels

For all genotypes, parasitemia ranged from 0.0001-0.1% (Fig. 5.5). However, there were significant differences in the parasitemia ranges between different parks. Parasitemia ranges for *T. parva* in the KNP and Hwange National Parks gave a wide range from 0.0001-0.1%. In contrast, parasitemia ranges in Marakele, Niassa (Mozambique) and Khaudum (Namibia) were much narrower (0.001-0.1%), while in Chobe National Park (Botswana) parasitemia were consistently below 0.01%. In Hluhluwe National Park, parasitemias were on average higher than the other parks (0.01-1%).

5.3.5 Competitive exclusion between different Theileriaparasites

A negative correlation was found for *T.* sp. (buffalo) and *T.* sp. (bougasvlei) at all localities (Table 5.2). In contrast, for mixed-infections with *T. parva*, both genotypes showed instances of low positive correlation, with an inverse negative correlation for the opposite genotype, again indicative of the negative association observed between *T.* sp. (buffalo) and *T.* sp. (bougasvlei).

Table 5.2: Correlation of co-occurrence of *Theileria* parasites. Indicated are the R*ij* values.

	Tpar/TspBuff	***Tpar/Bgvl***	***TspBuff/Bgvl***
KNP	0.031	-0.220	-0.737
KNP (South)	0.221	-0.713	-1.005
KNP (Mid)	-0.457	0.137	-0.573
KNP (North)	0.069	-0.163	-0.323
HGR	-0.057	-1.172	-0.736
CNP (Botswana)	-0.780	0.070	-0.771
MNP	0.127	-0.758	-0.770
HNP (Zimbabwe)	-0.469	0.102	-0.490
GNP (Zimbabwe)	-0.651	0.158	-0.651
GLTP (Sengwe corridor, Zimbabwe)	0.076	-0.226	-0.206
NNR (Mozambique)	-0.108	-1.09	-0.327
GLTP (Manguana Powerline, Mozambique)	0.072	-0.316	-0.435
KGR (Namibia)	-13.299	-0.787	-0.787
Diagnostic	-0.855	-1.57	-1.232

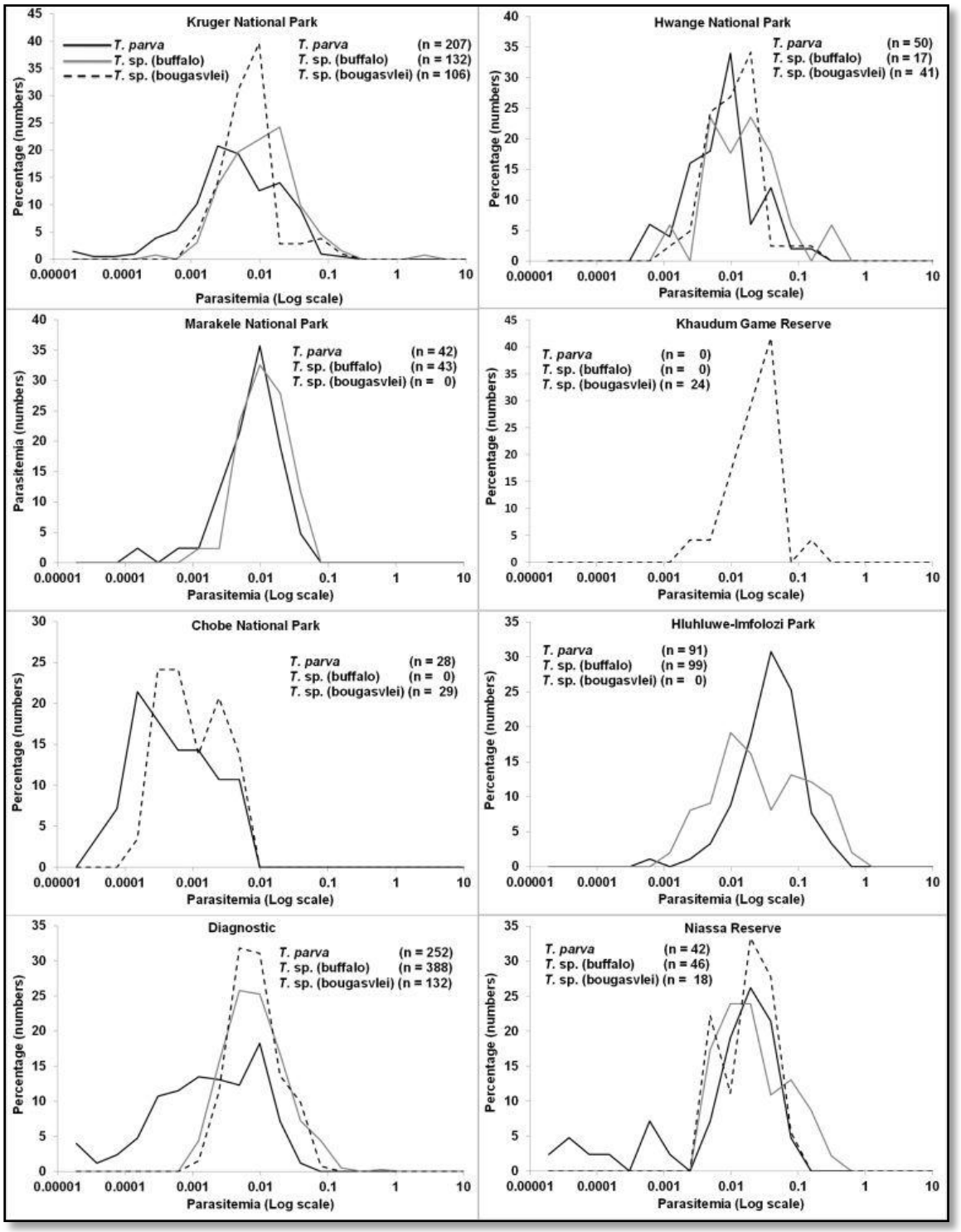


Figure 5.5: Parasitemia ranges for *T. parva*, *T.* sp. (buffalo) and *T.* sp. (bougasvlei) in different sample sets. Indicated are different National Parks as well as the diagnostic sample set. Number of data points for each genotype is indicated in brackets.

5.3.6 Ratios of mixed-infections

Expression of mixed-infections as parasitemia ratio, can be used to investigate whether any systematic skew exist, i.e. whether competitive exclusion was present. Analysis of animals with mixed-infections indicated that the parasitemia ranges for *T.* sp. (buffalo) and *T.* sp. (bougasvlei) spanned the normal range (Fig. 5.6A). A frequency plot of the parasitemia ratios approximates a normal distribution (Fig. 5.6B). No systematic skew could be observed and competitive exclusion between these genotypes within an animal with mixed-infections was not supported.

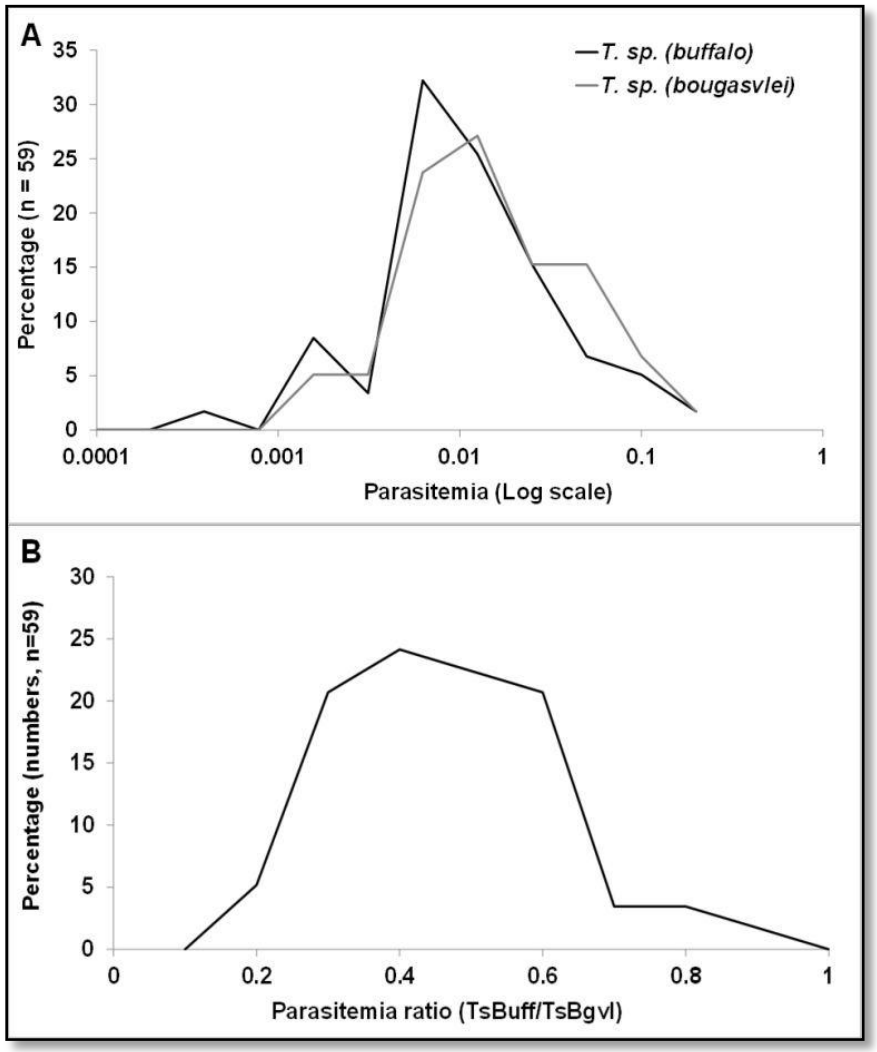
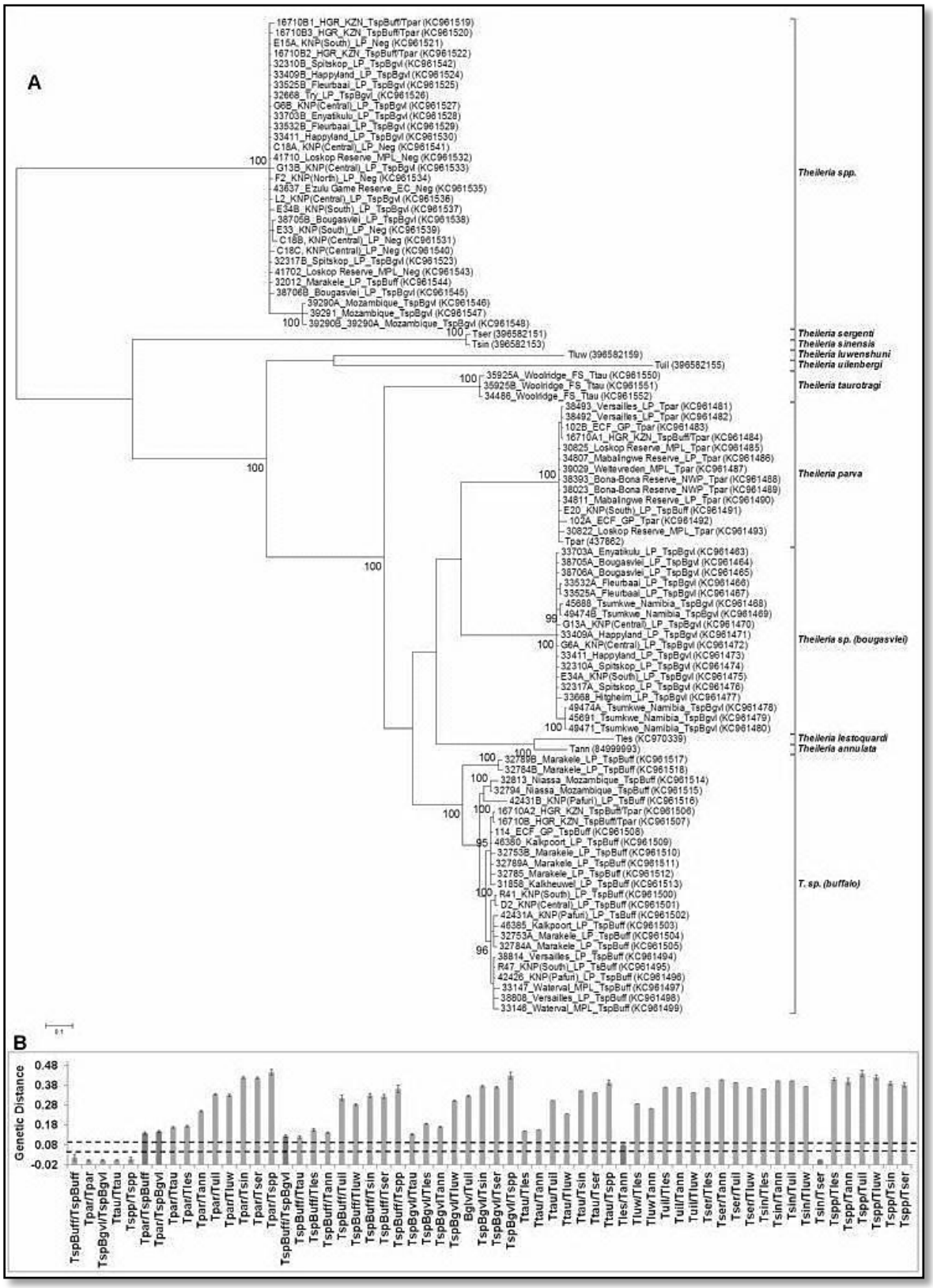


Figure 5.6: Parasitemia of animals with mixed-infections of *T.* sp. (buffalo) and *T.* sp. (bougasvlei). A) Parasitemia range of animals with mixed-infections. B) A frequency distribution of the parasitemia ratios of *T.* sp. (buffalo)/*T.* sp. (bougasvlei) for animals with mixed-infections.

5.3.7 *T. sp. (buffalo) and T. sp. (bougasvlei) as different species*

Phylogenetic analysis of COI indicated that all members of the "*T. taurotragi* clade" grouped as distinct genotypes (Fig. 5.7A). This included the known species *T. annulata*, *T. lestoquardi*, *T. parva* and *T. taurotragi*. *Theileria* sp. (bougasvlei) showed a closer genetic relationship to *T. parva* than *T.* sp. (buffalo) although with weak posterior probability support (83%). All samples from a specific genotype grouped within their respective clades with 100% posterior probability support with no support for the monophyly of *T.* sp. (buffalo) and *T.* sp. (bougasvlei). An unknown genotype grouped separately from any other *Theileria* genotype and based on a common genotype found in all the samples, could potentially represent *T. velifera*-B (unpublished observation, BJM). Analysis of the intra vs. inter-species genetic distances indicated that intra-species distances are all below 0.02 (Fig. 5.7B). In contrast the lowest inter-species distance was 0.079 for *T. annulata* and *T. lestoquardi*. Genetic distances between *T.* sp. (buffalo)/ *T.* sp. (bougasvlei), *T.* sp. (buffalo)/*T. parva* and *T. parva*/*T.* sp. (bougasvlei) were 0.124, 0.139 and 0.147, respectively. These values are similar to other inter-species distances within the "*T. taurotragi* clade" and well above the lowest distance for two recognized species, *T. annulata* and *T. lestoquardi*. This suggests that these genotypes represent different species and is supported by the fact that primers specific for *T.* sp. (buffalo) or *T.* sp. (bougsvlei) did not reciprocally amplify the other genotype. ABGD analysis separated the data into 9 groups after 9 recursive partitions, Group 1: *T.* sp. (buffalo), Group 2: *T. parva*, Group 3: *T.* sp. (bougasvlei), Group 4: *T. taurotragi*, Group 5: *T. annulata* and *T. lestoquardi*, Group 6: *T. luwenshuni*, Group 7: *T. uilenbergi*, Group 8: *T. sergenti* and *T. sinensis* and Group 9: *T.* spp. These correspond with the phylogenetic clades obtained during Bayesian analysis.

On page 103 - Figure 5.7: Bayesian analysis of the cytochrome oxidase (COI) gene. A) Sequences are indicated by an animal number, locality, province, *T. parva*, *T.* sp. (buffalo) or *T.* sp. (bougasvlei) test result and GenBank accession number in brackets. Posterior nodal support higher than 95% is indicated. B) Average genetic distances are indicated for intra- and inter-species with standard deviation as error bars. The upper broken line indicates the lowest limit considered for inter-species relationships as defined by *T. annulata* and *T. lestoquardi*. The lower broken line indicates the highest limit considered for intra-species relationships as defined by the upper deviation for *T.* sp. (buffalo). The distances between *T. parva*, *T.* sp. (buffalo) and *T.* sp. (bougasvlei) are indicated by dark grey bars.


A
16710B1_HGR_KZN_TspBuff/Tpar (KC961519)
16710B3_HGR_KZN_TspBuff/Tpar (KC961520)
E15A_KNP(South)_LP_Neg (KC961521)
16710B2_HGR_KZN_TspBuff/Tpar (KC961522)
32310B_Spitskop_LP_TspBgvl (KC961542)
33409B_Happyland_LP_TspBgvl (KC961524)
33525B_Fleurbaai_LP_TspBgvl (KC961525)
32668_Fry_LP_TspBgvl (KC961526)
G6B_KNP(Central)_LP_TspBgvl (KC961527)
33703B_Enyatikulu_LP_TspBgvl (KC961528)
33532B_Fleurbaai_LP_TspBgvl (KC961529)
33411_Happyland_LP_TspBgvl (KC961530)
C18A_KNP(Central)_LP_Neg (KC961541)
41710_Loskop Reserve_MPL_Neg (KC961532)
G13B_KNP(Central)_LP_TspBgvl (KC961533)
F2_KNP(North)_LP_Neg (KC961534)
43637_E'zulu Game Reserve_EC_Neg (KC961535)
L2_KNP(Central)_LP_TspBgvl (KC961536)
E34B_KNP(South)_LP_TspBgvl (KC961537)
38705B_Bougasvlei_LP_TspBgvl (KC961538)
E33_KNP(South)_LP_Neg (KC961539)
C18B_KNP(Central)_LP_Neg (KC961531)
C18C_KNP(Central)_LP_Neg (KC961540)
32317B_Spitskop_LP_TspBgvl (KC961523)
41702_Loskop Reserve_MPL_Neg (KC961543)
32012_Marakele_LP_TspBuff (KC961544)
38706B_Bougasvlei_LP_TspBgvl (KC961545)
39290A_Mozambique_TspBgvl (KC961546)
39291_Mozambique_TspBgvl (KC961547)
39290B_39290A_Mozambique_TspBgvl (KC961548)
Tser (396582151)
Tsin (396582153)
Tluw (396582159)
Tuil (396582155)
35925A_Woolridge_FS_Ttau (KC961550)
35925B_Woolridge_FS_Ttau (KC961551)
34486_Woolridge_FS_Ttau (KC961552)
38493_Versailles_LP_Tpar (KC961481)
38492_Versailles_LP_Tpar (KC961482)
102B_ECF_GP_Tpar (KC961483)
16710A1_HGR_KZN_TspBuff/Tpar (KC961484)
30825_Loskop Reserve_MPL_Tpar (KC961485)
34807_Mabalingwe Reserve_LP_Tpar (KC961486)
39029_Weltevreden_MPL_Tpar (KC961487)
38393_Bona-Bona Reserve_NWP_Tpar (KC961488)
38023_Bona-Bona Reserve_NWP_Tpar (KC961489)
34811_Mabalingwe Reserve_LP_Tpar (KC961490)
E20_KNP(South)_LP_TspBuff (KC961491)
102A_ECF_GP_Tpar (KC961492)
30822_Loskop Reserve_MPL_Tpar (KC961493)
Tpar (437862)
33703A_Enyatikulu_LP_TspBgvl (KC961463)
38705A_Bougasvlei_LP_TspBgvl (KC961464)
38706A_Bougasvlei_LP_TspBgvl (KC961465)
33532A_Fleurbaai_LP_TspBgvl (KC961466)
33525A_Fleurbaai_LP_TspBgvl (KC961467)
45688_Tsumkwe_Namibia_TspBgvl (KC961468)
49474B_Tsumkwe_Namibia_TspBgvl (KC961469)
G13A_KNP(Central)_LP_TspBgvl (KC961470)
33409A_Happyland_LP_TspBgvl (KC961471)
G6A_KNP(Central)_LP_TspBgvl (KC961472)
33411_Happyland_LP_TspBgvl (KC961473)
32310A_Spitskop_LP_TspBgvl (KC961474)
E34A_KNP(South)_LP_TspBgvl (KC961475)
32317A_Spitskop_LP_TspBgvl (KC961476)
33668_Hitgheim_LP_TspBgvl (KC961477)
49474A_Tsumkwe_Namibia_TspBgvl (KC961478)
45691_Tsumkwe_Namibia_TspBgvl (KC961479)
49471_Tsumkwe_Namibia_TspBgvl (KC961480)
Tles (KC970339)
Tann (84999993)
32789B_Marakele_LP_TspBuff (KC961517)
32784B_Marakele_LP_TspBuff (KC961518)
32813_Niassa_Mozambique_TspBuff (KC961514)
32794_Niassa_Mozambique_TspBuff (KC961515)
42431B_KNP(Pafuri)_LP_TsBuff (KC961516)
16710A2_HGR_KZN_TspBuff/Tpar (KC961506)
16710B_HGR_KZN_TspBuff/Tpar (KC961507)
114_ECF_GP_TspBuff (KC961508)
46380_Kalkpoort_LP_TspBuff (KC961509)
32753B_Marakele_LP_TspBuff (KC961510)
32789A_Marakele_LP_TspBuff (KC961511)
32785_Marakele_LP_TspBuff (KC961512)
31858_Kalkheuwel_LP_TspBuff (KC961513)
R41_KNP(South)_LP_TspBuff (KC961500)
D2_KNP(Central)_LP_TspBuff (KC961501)
42431A_KNP(Pafuri)_LP_TsBuff (KC961502)
46385_Kalkpoort_LP_TspBuff (KC961503)
32753A_Marakele_LP_TspBuff (KC961504)
32784A_Marakele_LP_TspBuff (KC961505)
38814_Versailles_LP_TspBuff (KC961494)
R47_KNP(South)_LP_TsBuff (KC961495)
42426_KNP(Pafuri)_LP_TspBuff (KC961496)
33147_Waterval_MPL_TspBuff (KC961497)
38808_Versailles_LP_TspBuff (KC961498)
33146_Waterval_MPL_TspBuff (KC961499)
Theileria spp.
Theileria sergenti
Theileria sinensis
Theileria luwenshuni
Theileria uilenbergi
Theileria taurotragi
Theileria parva
Theileria sp. (bougasvlei)
Theileria lestoquardi
Theileria annulata
T. sp. (buffalo)
B
Genetic Distance
0.48
0.38
0.28
0.18
0.08
-0.02
TspBuff/TspBuff
Tpar/Tpar
TspBgvl/TspBgvl
Ttau/Ttau
Tspp/Tspp
Tpar/TspBuff
Tpar/TspBgvl
Tpar/Ttau
Tpar/Tles
Tpar/Tann
Tpar/Tuil
Tpar/Tluw
Tpar/Tsin
Tpar/Tser
Tpar/Tspp
TspBuff/TspBgvl
TspBuff/Ttau
TspBuff/Tles
TspBuff/Tann
TspBuff/Tuil
TspBuff/Tluw
TspBuff/Tsin
TspBuff/Tser
TspBuff/Tspp
TspBgvl/Ttau
TspBgvl/Tles
TspBgvl/Tann
TspBgvl/Tluw
BgIv/Tuil
TspBgvl/Tsin
TspBgvl/Tser
TspBgvl/Tspp
Ttau/Tles
Ttau/Tann
Ttau/Tuil
Ttau/Tluw
Ttau/Tsin
Ttau/Tser
Ttau/Tspp
Tles/Tann
Tluw/Tles
Tluw/Tann
Tuil/Tles
Tuil/Tann
Tuil/Tluw
Tser/Tles
Tser/Tann
Tser/Tuil
Tser/Tluw
Tsin/Tles
Tsin/Tann
Tsin/Tuil
Tsin/Tluw
Tsin/Tser
Tspp/Tles
Tspp/Tann
Tspp/Tuil
Tspp/Tluw
Tspp/Tsin
Tspp/Tser

5.4 DISCUSSION

Distinction of species between genetically closely-related organisms remains problematic in the absence of unambiguous phenotypic traits. In the *Theileria*, phenotypic traits include microscopic morphology, vertebrate host or tick vector specificity, differential clinical disease outcomes or carrier-state biology (proliferation in the host and parasitemia), serological distinction and molecular markers. Few traits distinguish between *T.* sp. (buffalo) and *T.* sp. (bougasvlei). Morphologically no distinguishing features are known (Zweygarth et al. 2009). *Theileria* sp. (buffalo) was found in African buffalo in Uganda, Kenya and southern Africa, with no substantive reports that cattle can be infected (Oura et al. 2004; Oura et al. 2011; Pienaar et al. 2011a). *Theileria* sp. (bougasvlei) has only been found in African buffalo in southern Africa (Zweygarth et al. 2009; Mans et al. 2011a). Tick vectors for *T.* sp. (buffalo) and *T.* sp. (bougasvlei) remain unknown. Whereas *T. parva* is known to cause the related diseases of East Coast fever (ECF), January disease (Zimbabwe theileriosis) and Corridor disease in cattle, *T.* sp. (buffalo) and *T.* sp. (bougasvlei) are considered to be un-infective to cattle (Mans et al. 2011a; Mans et al. 2011b; Oura et al. 2011). While data regarding serological cross-reactivity is scarce, some strains of *T.* sp. (buffalo) might cross-react with *T. parva* antibodies and *vice versa* using IFAT or the PIM ELISA (Conrad et al. 1987; unpublished observations, AAL and BJM).

The only molecular differences exist in the V4-region of the 18S rRNA and the S5 ribosomal protein genes (Mans et al. 2011a). In the case of *T.* sp. (buffalo) and *T.* sp. (bougasvlei) there are 8 to10 nucleotide differences across the 18S rRNA gene and two indels (Chaisi et al. 2011). Between *T. parva* and *T.* sp. (bougasvlei), 10 to 14 nucleotide differences exist, and two indels. Nine to thirteen nucleotide differences exist between *T. parva* and *T.* sp. (buffalo), with no indels. For the S5 gene, *T. parva*, *T.* sp. (buffalo) and *T.* sp. (bougasvlei) gave similar pairwise genetic distances (Mans et al. 2011a). As such, molecular data do suggest that some form of differentiation exist between *T.* sp. (buffalo) and *T.* sp. (bougasvlei). The current study expands on this with regard to geographic distribution as well as the use of the mitochondrial COI gene as molecular marker.

Two novel assays, specific for *T.* sp. (buffalo) and *T.* sp. (bougasvlei) were developed and validated in the current study. Sensitivity of both assays compared well with the hybridization probe and Hybrid II assays for *T. parva* (Pienaar et al. 2011b). It is therefore possible to

distinguish these parasites in a single sample using different real-time PCR assays. It also indicates the potential of real-time PCR technology to derive quantitative data for animals with mixed *Theileria* infections.

Previously, 10% of KNP samples with mixed-infections showed PCR suppression of *T. parva* (Pienaar et al. 2011a). These samples were *T. parva* positive with the Hybrid II assay (Pienaar et al. 2011b), and are all *T.* sp. (buffalo) positive. No PCR suppression has been observed with *T.* sp. (bougasvlei). The parasitemia of *T.* sp. (bougasvlei) is generally lower than that of *T.* sp. (buffalo) and *T. parva*, while parasitemia for *T.* sp. (buffalo) is higher than that for *T. parva*. This could explain why mixed-infections with *T.* sp. (buffalo) lead to PCR suppression in a limited set of samples of the KNP (Pienaar et al. 2011a). No PCR suppression has been observed in the samples from Hluhluwe Game Reserve (HGR) where ~100% of buffalo possess mixed-infections of *T. parva* and *T.* sp. (buffalo). However, parasitemia in HGR is much higher for both parasites compared to the KNP, which could affect PCR suppression (Pienaar et al. 2011a). Parasitemia differences observed in National Parks may be related to tick density and park size which could impact on risk of PCR suppression.

Several different possibilities may explain the geographic distribution and parasitemia differences observed:

1) *Sampling bias.* In the case of diagnostic samples this could be a factor, since buffalo are commodities based on their *T. parva* disease-free status (Laubscher et al. 2012). Movement of buffalo infected with *T.* sp. (buffalo)-like parasites is not controlled and these could be moved to vector-free areas. Conversely, movement of buffalo into vector areas will promote the spread of the infection, thereby re-establishing historic endemic areas. However, the geographic distribution of diagnostic samples correlates with those from National Parks, where the parasites and vectors are assumed to be endemic. Sampling bias is therefore not considered to be a significant factor. Of interest was that RLB analysis previously indicated few *T.* sp. (buffalo) positive animals in the central KNP (Pienaar et al. 2011a). The current study explains this, as *T.* sp. (bougasvlei) predominates in the central KNP and will go undetected by RLB (Chaisi et al. 2011).
2) *PCR suppression due to mixed-infections.* It was previously shown that in at least 10% of cases in the KNP, suppression of PCR led to false-negative *T. parva* samples (Pienaar et al. 2011a). However, herd-wide suppression was never observed and suppression seems to

depend on individual parasitemia levels. This factor is therefore considered to be minor in the current study.

3) *Different tick vectors.* This could be a relevant factor, since the tick vectors for *T.* sp. (buffalo) and *T.* sp. (bougasvlei) remain unknown. If tick species play a significant role, this would support *T.* sp. (buffalo) and *T.* sp. (bougasvlei) being different species. Analysis of ticks found on buffalo in the KNP indicated that potential vectors could be *Amblyomma hebraeum*, *A. marmoreum*, *Hyalomma truncatum*, *R. appendiculatus*, *R. evertsi* or *R. simus* (Horak et al. 2007). Most of these ticks are distributed across all eco-zones in the park, even though some localized distributions are found (Spickett et al. 1991). As such, *R. zambeziensis* is localized in the northern and southern regions of the park, but not the central regions. This might suggest that it could be a vector of *T.* sp. (buffalo), however, it is not found in HGR.

4) *Host dispersal.* Movement of buffalo herds or individuals would explain localized distributions. However, the potential lifelong carrier state of *Theileria* in buffalo (the longest documented *T. parva* carrier period in a vector free area being twenty years (FT Potgieter, personal communication, 2013), the infection cycle of ticks that is dispersed over an annual period (which enhance contact with new hosts) and the fact that buffalo herds and individuals can migrate over large distances within short periods of time in response to environmental cues (Halley et al. 2002; Cross et al. 2005), would negate any host vicariance effects. As such, other *Theileria* species such as *T. parva*, *T. mutans* and *T. velifera* show no geographic localization as observed for *T.* sp. (buffalo) and *T.* sp. (bougasvlei) within the KNP.

5) *Gene duplicates in the same genome.* Thus far all *Theileria* species have two genomic copies of the 18S rRNA gene (Hayashida et al. 2012). It is therefore possible that the different genotypes observed might be different gene duplicates. This is relevant, since multiple divergent copies of the 18S gene has been identified in various protozoans, with the implication that the 18S rRNA gene is not useful to distinguish species compared to the COI gene (El-Sherry et al. 2013). However, if this was the case, co-detection would be the norm, while the COI gene phylogeny and ABGD analysis supports the designation as different species.

6) *Different alleles.* In this scenario, *T.* sp. (buffalo) and *T.* sp. (bougasvlei) would be considered to be the same species and the 18S variants different allelic forms (Chaisi et al. 2011). Differences in geographic distribution would be due to changes in allelic frequencies. The disparate geographic distribution of *T.* sp. (buffalo) and *T.* sp. (bougasvlei) would suggest that genetic drift played an important part in the fixation of alleles within the various geographic populations. This would suggest that small population sizes or bottleneck events are the norm in *Theileria* biology (McKeever, 2009). In this regard, *T. parva* was assumed to be panmictic, but has been shown to exhibit local sub-structuring in different populations (Oura et al. 2005; Muleya et al. 2012). The effective population size of a *Theileria* parasite in a buffalo with moderate parasitemia (0.01%) is, however, not small but in the range of 1 x 10^{10} parasites. Population bottlenecks certainly occur if it is considered that only ~1 x 10^{5} parasites are ingested by nymphal ticks, while less than a 1000 parasites infects the salivary glands, is subsequently transmitted to the host and that only one tick mating pairs survives (McKeever, 2009). A further bottleneck might occur due to host immunity in the case of heterologous challenge, where few parasites will mature to the point of piroplasms. However, given that the piroplasm carrier-state may be life long, that extensive recombination events do occur that make heterologous challenge more probable and that multiple tick challenges occur over many years, the reality of a bottleneck effect and small effective population sizes in a vector endemic region seems to be remote. The possibility that *T.* sp. (buffalo) and *T.* sp. (bougasvlei) belong to the same species is also not supported by either nuclear S5 ribosomal and mitochondrial COI gene phylogenies or ABGD analysis.
7) *Competitive exclusion of piroplasms in the buffalo host.* Competition of parasites for resources, specifically red blood cells in the host has been documented for anaplasmosis and babesiosis (Dib et al. 2008). The relatively low parasitemia levels observed for *T.* sp. (buffalo), *T.* sp. (bougasvlei) and *T. parva* do not, however, suggest that this is a significant mechanism utilized by these parasites, since multiple infections by *Theileria* genotypes seem to be the norm in African buffalo (Mans et al. 2011a). In addition, no skew was observed in parasitemia ratios for animals with mixed-infections.
8) *Immunological cross-reactivity.* If significant immunological cross-reactivity exists between species or strains, the establishment of carrier piroplasms could be prevented, since parasites will not have time to mature before elimination by the host's immune system. In such a

scenario, the piroplasm population might be established during the initial infection, while subsequent infections will not contribute towards genetic diversity. This scenario seems unlikely, given that heterologous strains exist and are known to be responsible for re-infection (McKeever, 2009).

9) *Genetic incompatibility.* This would imply that *T.* sp. (buffalo) and *T.* sp. (bougasvlei) are closely related but different *Theileria* species. In this scenario, these species recently diverged and is transmitted via the same tick vector. A feeding tick that ingests parasites from a buffalo with mixed-infections, results in mating, genetic incompatibility and/or sterile hybrid progeny (Coyne and Orr, 2004). Mixed-infections in the vertebrate host will therefore adversely affect population fitness, with a resulting fixation of a specific species in the buffalo population. In areas where buffalo migrate and come into contact with their opposite carriers, mixed-infections will occur until fixation of a specific genotype occurs. Such zones will be found wherever migrating buffalo meet. In the present study, the region in Northern KNP is of interest, since the Transfrontier Park has opened up in 2008 with the Senge Corridor linking the KNP and Goranguru National Park. Migration and contact of buffalo in this region is therefore possible and creates an unstable zone where mixed-infections will occur. This does not imply that mixed-infections in the buffalo host cannot be maintained in the carrier-state, but do suggest that such buffalo might be dead-end hosts for the parasites. This mechanism will allow parasites to mimic geographical isolation within a shared vertebrate host and tick vector and ensure speciation. Once mating does not occur anymore, mixed-infections will not be problematic. Once the tick vector for *T.* sp. (buffalo) and *T.* sp. (bougasvlei) has been identified, it would be possible to test this hypothesis on a more formal basis, by tick transmission experiments. It also implies that *T.* sp. (bougasvlei) and *T. parva*, which seem to be genetically closer related, have different tick vectors and hybrid sterility would therefore not be problematic. The possibility that hybridization may occur between *T.* sp. (buffalo) and *T.* sp. (bougasvlei) exist. However, co-segregation of nuclear (18S) and mitochondrial (COI) markers do not support this possibility.

In the case of *T. parva* and *T. taurotragi*, which shares the same tick vector and cattle as vertebrate hosts, but also specifically infect buffalo and eland, respectively (Bishop et al. 2004; Oura et al. 2011), host specificity as means of geographic isolation probably played a larger role

than genetic incompatibility. This implies that speciation occurred in these species before introduction of cattle into Africa. Mechanisms for speciation in *Theileria* could therefore include host and tick vector specificity as well as genetic incompatibility.

CHAPTER 6

General Conclusions

The current study confirmed the hypothesis that mixed-infections affect the current diagnostic real-time hybridization assay for *T. parva*. This was achieved by the use of independent protein marker assays that showed that the current real-time assay fail to detect all *T. parva* positive samples. Using a unique strategy to quantify parasitemia levels in carrier buffalo from real-time PCR results, parasitemia ranges for *T. parva* and *T.* sp. (buffalo)-like parasites in buffalo could be determined. This allowed modelling of mixed-infections which showed that false-negative results may be obtained when *T. parva* parasitemia is low in the carrier host and the parasitemia ratio of *T.* sp. (buffalo) to *T. parva* is high. This leads to PCR suppression of the amplification and melting peak signals to yield results that resemble *T.* sp. (buffalo)-like positive samples.

To address PCR suppression, a number of different assays were developed and validated. These include assays independent of the 18S rRNA gene based on protein markers as well as a new assay (Hybrid II) that utilize the 18S rRNA gene but is not affected by mixed infections. These assays show promise to replace the current test in the future, especially if the geographic distribution of *T.* sp. (buffalo)-like parasites increases. In this regard, novel real-time PCR hybridization assays specific for *T.* sp. (buffalo) and *T.* sp. (bougasvlei) were developed that allowed the determination of which parasite affects the current real-time PCR assay.

PCR suppression is mostly due to *T.* sp. (buffalo), since its parasitemia is generally higher than that observed for *T.* sp. (bougasvlei) and it was the only parasite present in false-negative samples. These parasites were shown to be closely related but distinct species by analysis of the COI gene. Observation of a low mixed-infection incidence for these respective parasites led to the hypothesis that they are potentially transmitted by the same tick vector where they can mate, leading to sterile hybrid progeny, which explains peculiar distribution in carrier buffalo.

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