CHAPTER 3

Development and evaluation of a real-time PCR test for detection of *Theileria parva* infections in Cape buffalo (*Syncerus caffer*) and cattle

Whatsoever thy hand findeth to do, do it with thy might; for there is no work, nor device, nor knowledge, nor wisdom, in the grave, whither thou goest. ***Ecclesiastes 9:10***

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"The journey of a thousand miles begins with one step." ***Lao-Tse***
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3.1 Abstract

Corridor disease, caused by the tick-borne protozoan parasite *Theileria parva*, is a controlled disease in South Africa. The Cape buffalo (*Syncerus caffer*) is the reservoir host and uninfected buffalo have become sought-after by the game industry in South Africa, particularly for introduction into Corridor disease-free areas. A real-time polymerase chain reaction (PCR) test for detection of *T. parva* DNA in buffalo and cattle was developed to improve the sensitivity and specificity of the official diagnostic test package in South Africa. Oligonucleotide primers and hybridization probes were designed based on the 18S ribosomal RNA (rRNA) gene. Amplification of control DNA using *Theileria* genus-specific primers resulted in detection of *T. taurotragi* and *T. annulata,* in addition to *T. parva.* A *T. parva*-specific forward primer was designed which eliminated amplification of all other *Theileria* species, except for *Theileria* sp. (buffalo); however only the *T. parva* product was detected by the *T. parva*-specific hybridization probe set. The real-time PCR assay requires less time to perform, is more sensitive than the other molecular assays previously used in *T. parva* diagnostics and can reliably detect the parasite in carrier animals with a piroplasm parasitaemia as low as 8.79×10^{-4}%.

3.2 Introduction

Theileria parva is a tick-borne protozoan parasite which occurs in eastern, central and southern Africa, and causes East Coast fever (ECF), Corridor disease and January disease (Uilenberg *et al.*, 1982; Perry *et al.*, 1991). The Cape buffalo (*Syncerus caffer*) is the natural reservoir of the parasite. Buffalo are also carriers of several other *Theileria* species which can infect cattle, including the relatively benign *T. mutans* and the apathogenic *T. velifera*. Under certain circumstances another group of relatively benign organisms, belonging to the *T. buffeli* / *T. orientalis* / *T. sergenti* complex can also cause disease in cattle and loss of production (Norval *et al.*, 1992). *Theileria buffeli* and *Theileria* sp. (buffalo) have been identified in some buffalo populations in South Africa (Stoltsz, 1996). Very little is known about *Theileria* sp. (buffalo) which was first recognised in an isolate from a buffalo in Kenya (Allsopp *et al.*, 1993). The eland (*Taurotragus oryx*) is the reservoir host of *T. taurotragi*, which can also infect cattle and has been implicated in bovine cerebral theileriosis in South Africa (De Vos *et al.*, 1981). *Theileria annulata,* which causes tropical theileriosis in cattle in other parts of the world, does not occur in South Africa.

Theileria parva infection of cattle is a controlled disease in South Africa. Transmission of the parasite to cattle by three species of ticks, *Rhipicephalus appendiculatus, R. zambeziensis* and *R. duttoni,* causes a form of theileriosis known as Corridor disease (Neitz, 1955; Neitz, 1957; Blouin and Stoltsz, 1989). Corridor disease is an acute, usually fatal disease of cattle resembling ECF. The disease is characterized by the proliferation of lymphoblasts infected with theilerial schizonts throughout the body, particularly in the lymph nodes, lymphoid aggregates, spleen, kidneys, liver and lungs. Contrary to ECF, the course of the disease is usually shorter, death occurring only three to four days after the onset of first clinical signs (Lawrence *et al*., 1994). Transmission of the disease occurs in cattle sharing grazing grounds with infected buffalo in the presence of the tick vector, resulting in buffalo to cattle transmission. The South African cattle population is completely naïve to *T. parva* infection and should be protected against exposure. Therefore, contact between infected buffalo in game reserves and cattle, is strictly controlled by the veterinary authorities (Stoltsz, 1989).

In recent years, there has been an increased risk of theileriosis in South Africa through the breeding and translocation of so-called “disease-free” buffalo, i.e. buffalo that test negative for foot-and-mouth disease (FMD), bovine brucellosis, bovine tuberculosis and Corridor disease. Buffalo must be tested for these diseases before they are allowed to be relocated throughout the country. In the case of Corridor disease, depending on the origin and history of buffalo, buffalo must undergo one to five tests, the indirect immunofluorescent antibody (IFA) test and PCR/probe assay each time, before they are allowed to be moved or certified disease free as determined by the Veterinary authorities. With the expansion of the game industry in South Africa in the 1990s, “disease-free” buffalo have become a sought-after commodity, particularly for introduction into Corridor disease-free areas. The movement of buffalo from the large, genetically diverse herds in the Kruger National Park is prohibited as FMD, bovine tuberculosis and Corridor disease are endemic there. The relocation of buffalo from the KwaZulu-Natal parks is also prohibited as Corridor disease is endemic there. The major source of animals free from these diseases has previously been a relatively small herd at the Addo Park in the eastern Cape (Stoltsz, 1989). However, there are not enough animals at Addo to meet the increased demand and the stock is limited in genetic diversity. This led to the establishment of buffalo breeding projects from infected parent stock, some of them in areas where the vector ticks for Corridor disease occur.

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A further area of concern is the possibility of the creation of *T. parva* carrier cattle. If *T. parva* infected cattle are treated or recover spontaneously, they may become carriers of the parasite (Potgieter *et al*., 1985; Dolan, 1986; Maritim *et al*., 1989; Kariuki *et al*., 1995; Marcotty *et al*., 2002). Ticks can acquire infections from carrier cattle and a situation could eventually develop where the parasite becomes adapted to cattle as hosts, resulting in cattle to cattle transmission, as appears to have happened with East Coast fever and January disease (Potgieter *et al*., 1988). Accurate diagnostic tests are therefore required in South Africa to identify infected buffalo and cattle and to assist the veterinary regulatory authorities to control the movement of buffalo.

Conventional diagnosis of *T. parva* is based on the microscopic demonstration of schizonts in lymphocytes, piroplasms in erythrocytes, clinical signs and pathology as well as detection of serum antibodies to schizont antigens, using the (IFA) test (Brocklesby and Barnett, 1966; Burridge *et al*., 1973; Burridge *et al*., 1974; Radley *et al*., 1974; Goddeeris *et al*., 1982). It is impossible to differentiate *T. parva* schizonts and piroplasms from most other *Theileria* spp. using light microscopy. Disadvantages of the IFA test include cross-reactivity between certain species, difficulty in standardization and subjectivity in interpretation of the results (Norval *et al*., 1992). In addition, antibodies may not be detected if the animal is not subject to a continuous tick challenge (Burridge and Kimber, 1972). Several molecular techniques for diagnosing *Theileria* infections have therefore been developed involving the use of the polymerase chain reaction (PCR) and DNA probes (Bishop *et al*., 1992; Allsopp *et al*., 1993; Bishop *et al*., 1995; Gubbels *et al*., 1999; Collins *et al*., 2002; Ogden *et al*., 2003). These techniques have improved the sensitivity and specificity that previous diagnostic tests lacked. However, PCR and probing assays are relatively time-consuming and labour intensive, particularly when separate hybridization steps are required to confirm test outcomes. There is therefore a need for a rapid, more sensitive and specific diagnostic test to accurately detect *T. parva* infections in buffalo and cattle.

Recently, real-time PCR technology has greatly improved molecular detection of organisms of veterinary, medical and economic importance (Nicolas *et al*., 2002; Moonen *et al*., 2003; Stone *et al*., 2004; Kares *et al*., 2004; Orrù *et al*., 2004; Whiley *et al*., 2004; Bischoff *et al*., 2005; Kim *et al*., 2005; Ramaswamy *et al*., 2005). This technique enables the accurate detection and quantification of specific DNA in various biological samples and also allows differentiation of species or strains of important pathogenic organisms. The use of the LightCycler® (Roche Diagnostics, Mannheim, Germany) allows fast real-time monitoring of a

PCR, where amplification and detection can be accomplished in a closed capillary tube, minimizing contamination problems. Therefore real-time PCR technology was chosen for the development of a rapid, sensitive and specific assay for detection of *T. parva*.

3.3 Materials and methods

3.3.1 Sample collection

Cattle and buffalo blood samples from different areas in South Africa were investigated. These included three known *T. parva* positive and 55 negative samples as well as 309 field samples of unknown status (Table 3.1). Gold standard positive samples included a naturally infected buffalo (KNP102 donated by South African National Parks) and two experimentally infected cattle, 9288 (splenectomized) and 9445 (intact). Both cattle (9288 and 9445) were infected with the Welgevonden *T. parva* isolate, which originated from two buffalo (welg23/04 and welg24/04) from the Welgevonden Private Game Reserve, located in the Limpopo Province of South Africa. The buffalo tested positive for *T. parva* using standard PCR (Allsopp *et al*., 1993) and reverse line blot (RLB) hybridization (Gubbels *et al*., 1999) tests. Bovine 9288 was infected with *T. parva* using *R. appendiculatus* adults of which the nymphal stage fed on buffalo welg23/04 and welg24/04 to pick-up the infection (xenodiagnosis). The animal reacted severely but recovered without treatment. Subsequently laboratory-reared *R. appendiculatus* nymphs were fed on it and the ensuing adult ticks were placed on Bovine 9445, which developed classical Corridor disease and died. Fifty-five fully susceptible cattle bred, reared and maintained under tick-free conditions for the purpose of live-blood vaccine production, at the Agricultural Research Council-Onderstepoort Veterinary Institute (ARC-OVI), South Africa, were used as gold standard negative samples.

Table 3.1 Origin and number of samples used for the evaluation of the *T. parva* real-time PCR test

Sample type	Origin of samples	Number of blood samples and animal of origin
Gold standard positive	Kruger National Park (KNP Welgevonden Game Reserve	1 buffalo (KNP102) 2 cattle [9288 (splenectomized), 9445 (intact)]*
Gold standard negative	ARC-OVI	55 cattle
Field	KNP Hluhluwe-iMfolozi Game Reserve Ladysmith farm Mabalingwe Game Reserve Marekele National Park Bloemfontein Kaalplaas farm ARC-OVI	65 buffalo 41 buffalo 34 cattle 6 buffalo and 6 cattle 15 buffalo 1 bovine 34 cattle 107 buffalo and cattle

*Experimentally infected cattle

3.3.2 DNA extraction

All DNA extractions were performed using the High Pure PCR template preparation kit (Roche Diagnostics, Mannheim, Germany) from 200 µl of EDTA blood samples. Extracted DNA was eluted in 100 µl elution buffer and stored at 4°C until further analysis.

3.3.3 Design of primers and hybridization probes

Theileria genus-specific forward [5'-GGT AAT TCC AGC TCC AAT AG-3'] and reverse [5'-ACC AAC AAA ATA GAA CCA AAG TC-3'] primers were designed for amplification of a 230 bp fragment of the V4 variable region of the 18S rRNA gene from all *Theileria* species (Figure 3.1). In addition, a forward primer [5'-CTG CAT CGC TGT GTC CCT T-3'] for specific amplification of *T. parva* was designed. For the specific detection of *T. parva* amplicons, a pair of hybridization probes [*T. parva* anchor: 5'-GGG TCT CTG CAT GTG GCT TAT--FL; *T. parva* sensor: 5'-LCRed640-TCG GAC GGA GTT CGC T—PH] was designed complementary to a *T. parva*-specific region within the amplicon (Figure 3.1). For the detection of the presence of any *Theileria* species in a sample, a pair of hybridization probes was selected complementary to a region conserved between nine *Theileria* species for which 18S rRNA gene sequence data is known (*T. annulata*_AY150056, *T. annulata*_M64243, *T. lestoquardi*_AF081135, *T. parva*_L28999, *T. parva*_L02366, *T. parva*_AF013418, *Theileria* sp. (buffalo) (Allsopp *et al.*, 1993), *T. taurotragi*_L19082, *T. buffeli*_AF236094, *T. buffeli*_Warwick-Australia_AB000272, *T. sergenti*_AY661514, *T. buffeli*_DQ104611, *T. buffeli*_AF236097, *T. buffeli*_Z15106, *T. sergenti*_AB016074,

*T. sergenti*_AF081137, *T. velifera*_AF097993, *T. mutans*_AF078815) [*Theileria* genus anchor: 5'-AGA AAA TTA GAG TGC TCA AAG CAG GCT TT--FL; *Theileria* genus sensor: 5'-LCRed705-GCC TTG AAT AGT TTA GCA TGG AAT—PH] (Figure 3.1). All primers and fluorescently-labelled hybridization probes were synthesized by TIB Molbiol (Berlin, Germany).

3.3.4 Optimized real-time PCR conditions

Amplification mixtures consisted of 4 µl of 10x LightCycler-FastStart DNA Master^{Plus} Hybridization Probes mix, yielding a final concentration of 2x in 20 µl total volume (Roche Diagnostics, Mannheim, Germany), 0.5 µM of each primer, 0.1 µM of each hybridization probe, 1U uracil deoxy-glycosylase (UDG) (Roche Diagnostics, Mannheim, Germany) and 1 to 2.5 µl (~15 ng to ~37.5 ng) of template DNA in a final volume of 20 µl. Temperature cycling was performed in a LightCycler® v2 (Roche Diagnostics, Mannheim, Germany). The UDG was activated at 40°C for 10 min before the FastStart *Taq* DNA polymerase activation step of 10 min at 95°C. The amplification programme included 45 cycles of three steps each, comprising denaturing at 95°C for 10 sec, primer annealing at 58°C for 10 sec, and product extension at 72°C for 15 sec. Following amplification, a melting curve analysis was performed by heating the samples from 40°C to 95°C with a heating rate of 0.2°C/sec. Fluorescence values were measured at 640 and 705 nm.

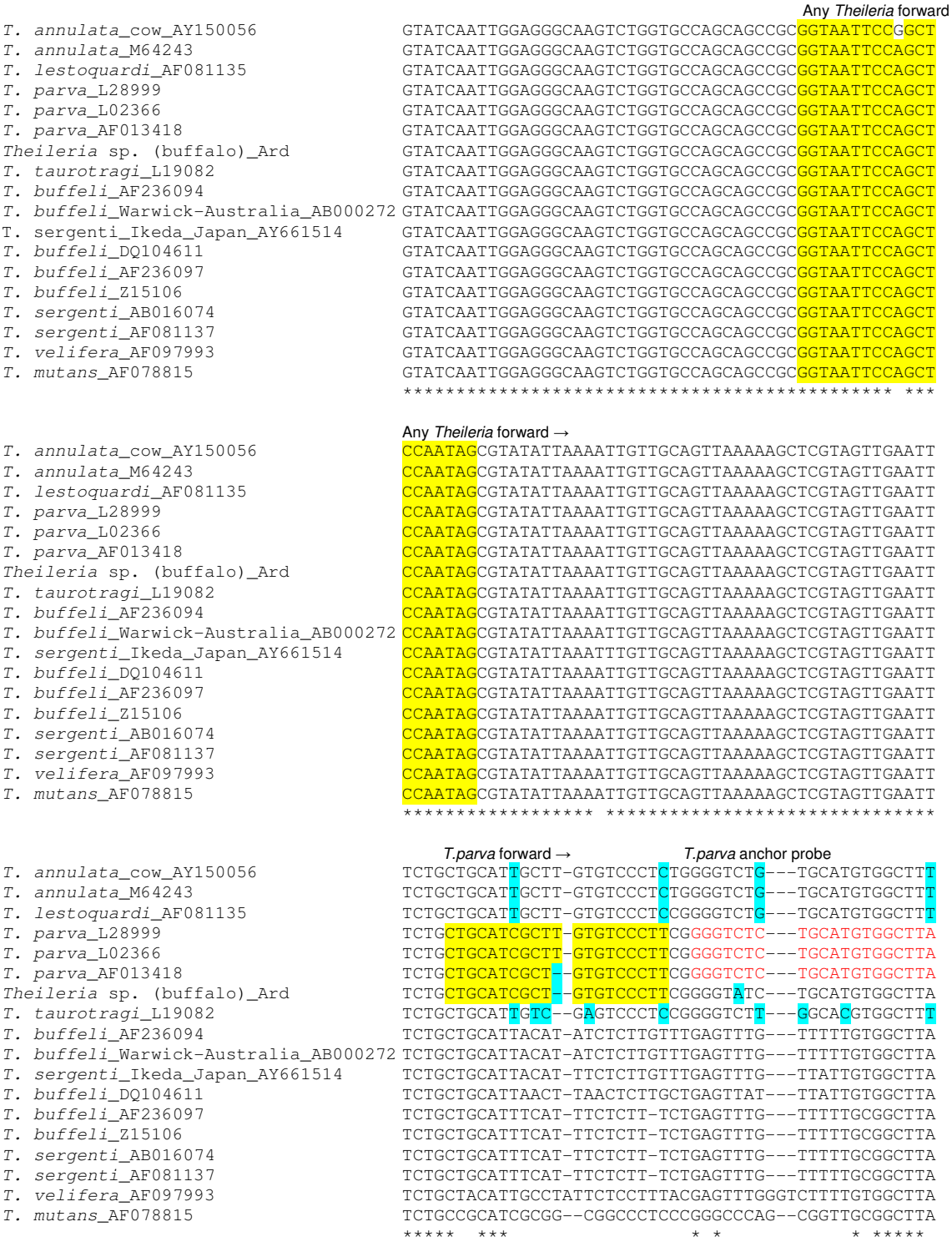

Figure 3.1 CLUSTAL X (1.81) multiple sequence alignment of the V4 variable region of published *Theileria* 18S rRNA gene sequences. Accession numbers for each sequence are provided. Amplification primers are highlighted in yellow. *T. parva*-specific hybridization probes are coloured in red, and hybridization probes designed to detect the presence of any *Theileria* species are in blue. Differences between the *T. parva* sequence and the four most closely related species (*T. annulata*, *T. lestoquardi*, *Theileria* sp. (buffalo), and *T. taurotragi*) are highlighted in cyan. This figure continues on page 49.

T.parva sensor probe

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T. annulata_cow_AY150056                TTTCGGACGGAGTTT-CTTTGTCTGAATGTTTACTTAGAGAAAAT-AGAG
T. annulata_M64243                      TTTCGGACGGAGTTT-CTTTGTCTGAATGTTTACTTTGAGAAAATTAGAG
T. lestoquardi_AF081135                 TTTCGGACGGAGTTT-CTTTGTCTGAATGTTTACTTTGAGAAAATTAGAG
T. parva_L28999                         TTTCGGACGGAGTTCGCTTTGTCTGGATGTTTACTTTGAGAAAATTAGAG
T. parva_L02366                         TTTCGGACGGAGTTCGCTTTGTCTGGATGTTTACTTTGAGAAAATTAGAG
T. parva_AF013418                       TTTCRGACGGAGTTCGCTTTGTCTGGATGTTTACTTTGAGAAAATTAGAG
Theileria sp. (buffalo)_Ard             TTTCAGACGGAGTTTACTTTGTCTGGATGTTTACTTTGAGAAAATTAGAG
T. taurotragi_L19082                    TTTCGGACGG--TTCGCT--GTCTGGATGTTTACTTTGAGAAAATTAGAG
T. buffeli_AF236094                     TTTCGGTTTGATTTTT-TCTTTCCGGATGATTACTTTGAGAAAATTAGAG
T. buffeli_Warwick-Australia_AB000272   TTTCGGTTTGATTTTT-TCTTTCCGGATGATTACTTTGAGAAAATTAGAG
T. sergenti_Ikeda_Japan_AY661514        TTTCGGATTGATTTTTATCATTCCGGATGATTACTTTGAGAAAATTAGAG
T. buffeli_DQ104611                     TTTCGGATTGATTTTT-TCTTTCCGGATGATTACTTTGAGAAAATTAGAG
T. buffeli_AF236097                     TTTCGGTTTGATTTTT-TCTTTCCGGATGATTACTTTGAGAAAATTAGAG
T. buffeli_Z15106                       TTTCGGTTTGATTTTT-TCTTTCCGGATGATTACTTTGAGAAAATTAGAG
T. sergenti_AB016074                    TTTCGGTTTGATTTTT-TCTTTCCGGATGATTACTTTGAGAAAATTAGAG
T. sergenti_AF081137                    TTTCGGTTTGATTTTT-TCTTTCCGGATGATTACTTTGAGAAAATTAGAG
T. velifera_AF097993                    TCTGGGTTCGCTTGCT----TCCCGGTGTTTTACTTTGAGAAAATTAGAG
T. mutans_AF078815                      TTTCGGACTCGCTTGC--GTCTCCGAATGTTTACTTTGAGAAAATTAGAG
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Any *Theileria* anchor probe Any Theileria sensor probe

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T. annulata_cow_AY150056                TGCTCAAAGCAGGCTTTCGCCTTGAATAGTTTAGCATGGAATAATAAAGT
T. annulata_M64243                      TGCTCAAAGCAGGCTTTCGCCTTGAATAGTTTAGCATGGAATAATAAAGT
T. lestoquardi_AF081135                 TGCTCAAAGCAGGCTTTTGCCTTGAATAGTTTAGCATGGAATAATAGAGT
T. parva_L28999                         TGCTCAAAGCAGGCTTTTGCCTTGAATAGTTTAGCATGGAATAATAAAGT
T. parva_L02366                         TGCTCAAAGCAGGCTTTTGCCTTGAATAGTTTAGCATGGAATAATAAAGT
T. parva_AF013418                       TGCTCAAAGCAGGCTTTTGCCTTGAATAGTTTAGCATGGAATAATAAAGT
Theileria sp. (buffalo)_Ard             TGCTCAAAGCAGGCTTTTGCCTTGAATAGTTTAGCATGGAATAATAAAGT
T. taurotragi_L19082                    TGCTCAAAGCAGGCTTTTGCCTTGAATAGTTTAGCATGGAATAATAAAGT
T. buffeli_AF236094                     TGCTCAAAGCAGGCTTTTGCCTTGAATAGTTTAGCATGGAATAATAAAGT
T. buffeli_Warwick-Australia_AB000272   TGCTCAAAGCAGGCTTTTGCCTTGAATAGTTTAGCATGGAATAATAAAGT
T. sergenti_Ikeda_Japan_AY661514        TGCTCAAAGCAGGCTTTTGCCTTGAATAGTTTAGCATGGAATAATAGAGT
T. buffeli_DQ104611                     TGCTCAAAGCAGGCTTTTGCCTTGAATAGTTTAGCATGGAATAATAAAGT
T. buffeli_AF236097                     TGCTCAAAGCAGGCTTTTGCCTTGAATAGTTTAGCATGGAATAATAAAGT
T. buffeli_Z15106                       TGCTCAAAGCAGGCTTTTGCCTTGAATAGTTTAGCATGGAATAATAAAGT
T. sergenti_AB016074                    TGCTCAAAGCAGGCTTTTGCCTTGAATAGTTTAGCATGGAATAATAAAGT
T. sergenti_AF081137                    TGCTCAAAGCAGGCTTTTGCCTTGAATAGTTTAGCATGGAATAATAAAGT
T. velifera_AF097993                    TGCTCAAAGCAGGCTTTTGCCTTGAATAGTTTAGCATGGAATAATAAAGT
T. mutans_AF078815                      TGCTCAAAGCAGGCCTTGCCTTGAATACTTTAGCATGGAATAATAAAGT
                                        ************** * ********** ***************** ***
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← Any *Theileria* reverse

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T. annulata_cow_AY150056                AGGACTTTGGTTCTATTTTGTTGGTT
T. annulata_M64243                      AGGACTTTGGTTCTATTTTGTTGGTT
T. lestoquardi_AF081135                 AGGACTTTGGTTCTATTTTGTTGGTT
T. parva_L28999                         AGGACTTTGGTTCTATTTTGTTGGTT
T. parva_L02366                         AGGACTTTGGTTCTATTTTGTTGGTT
T. parva_AF013418                       AGGACTTTGGTTCTATTTTGTTGGTT
Theileria sp. (buffalo)_Ard             AGGACTTTGGTTCTATTTTGTTGGTT
T. taurotragi_L19082                    AGGACTTTGGTTCTATTTTGTTGGTT
T. buffeli_AF236094                     AGGACTTTGGTTCTATTTTGTTGGTT
T. buffeli_Warwick-Australia_AB000272   AGGACTTTGGTTCTATTTTGTTGGTT
T. sergenti_Ikeda_Japan_AY661514        AGGACTTTGGTTCTATTTTGTTGGTT
T. buffeli_DQ104611                     AGGACTTTGGTTCTATTTTGTTGGTT
T. buffeli_AF236097                     AGGACTTTGGTTCTATTTTGTTGGTT
T. buffeli_Z15106                       AGGACTTTGGTTCTATTTTGTTGGTT
T. sergenti_AB016074                    AGGACTTTGGTTCTATTTTGTTGGTT
T. sergenti_AF081137                    AGGACTTTGGTTCTATTTTGTTGGTT
T. velifera_AF097993                    AGGACTTTGGTTCTATTTTGTTGGTT
T. mutans_AF078815                      AGGACTTTGGTTCTATTTTGTTGGTT
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3.3.5 Specificity of the real-time PCR assay

To determine the analytical specificity of the real-time PCR assay, 2.5 µl (~37.5 ng) DNA of several different *Theileria* species, including *T. annulata, T. taurotragi, T. velifera, T. buffeli, T. mutans*, *Theileria* sp. (buffalo) and *T. parva* was subjected to the assay. In addition, DNA from other blood parasites including *Babesia* spp., *Ehrlichia* spp., *Anaplasma* spp., *Trypanosoma* spp. and bacteria commonly found in cattle and buffalo were tested for this purpose (Table 3.2). DNA from the 55 gold standard negative cattle (Table 3.1) was also tested. Both sets of primers (the *T. parva*-specific forward primer together with the *Theileria* genus-specific reverse primer and the *Theileria* genus-specific forward primer together with the *Theileria* genus-specific reverse primer) were used in separate amplification reactions and both probe sets were included in the reactions for detection of PCR products.

Table 3.2 Specificity of the *T. parva* real-time PCR test using the *T. parva*-specific forward primer, the *Theileria* genus-specific reverse primer and both probe sets

Sample	Results			
	Amplification		Melting peak (temperature)	
	***T. parva* 640 nm**	***Theileria* species 705 nm**	***T. parva* 640 nm**	***Theileria* species 705 nm**
Theileria parva (KNP102)	+	+	+	+
Theileria sp. (buffalo)	(+)	+	-	+ (63°C)
Theileria buffeli + *Theileria mutans* (14044)	-	-	-	-
Theileria mutans (14043)	-	-	-	-
Theileria taurotragi + *Theileria mutans* + *Theileria parva* (14048)	+	+	+ (63°C)	+ (62°C)
Theileria taurotragi + *Theileria mutans* + *Theileria parva* (14049)	+	+	+ (63°C)	+ (62°C)
Theileria taurotragi + *Theileria mutans* (14045)	-	-	-	-
Theileria taurotragi + *Theileria mutans* (14046)	-	-	-	-
Theileria taurotragi + *Theileria mutans* (14047)	-	-	-	-
Arcanobacterium pyogenes Bacteria 6964/1B (1)	-	-	-	-
Bacillus lactosporus Bacteria 9879/2(2)	-	-	-	-
Staphyloccocus aureus Bacteria 9351/1(3)	-	-	-	-
Escherichia coli Bacteria 097(4)	-	-	-	-
Salmonella typhimurium Bacteria1021/6(5)	-	-	-	-
Enterococcus facium Bacteria 9351/3(6)	-	-	-	-
Ehrlichia ruminantium (Ball3 vaccine strain) Heartwater-5540 (16928)	-	-	-	-
Ehrlichia ruminantium (Ball3 vaccine strain) Heartwater-5244 (16929)	-	-	-	-
Anaplasma centrale-8230 (16931)	-	-	-	-
Babesia bigemina + *A. centrale*-9456.1 (16932)	-	-	-	+ (48.8)*
Babesia bovis (16823)	-	-	-	-
Babesia bigemina (16824)	-	-	-	+ (48.8)*
Theileria equi-20 (16369)	-	-	-	-
Babesia caballi- 502 (16368)	-	-	-	-
Trypanosome-29b (16367)	-	-	-	-
Trypanosome -27b (16366)	-	-	-	-
Trypanosome -24b (16365)	-	-	-	-
Trypanosome -8a (16363)	-	-	-	-

*Although no amplification was observed in these samples, a melting peak at 48.8°C was detected at 705 nm. This probably indicates that a non-specific PCR product was obtained from *Babesia bigemina* DNA, and the *Theileria* genus-specific hybridization probes were able to anneal to this product, yielding a melting peak at 48.8°C. The *T. parva*-specific probes did not hybridize to this non-specific product.

3.3.6 Sensitivity of the real-time PCR assay

To determine the lower limit of detection of the real-time PCR assay for *T. parva*, blood from a naturally infected buffalo (KNP102) with a piroplasm parasitaemia of 0.009% was used. The parasitaemia was determined by examining approximately 34000 erythrocytes and counting the number of infected cells. The *T. parva* infection status of the buffalo was confirmed by performing a xenodiagnosis. Approximately 1200 *R. appendiculatus* nymphs were fed on buffalo KNP102 in ear-bags. After 5 days, approximately 450 ticks were collected; the engorged nymphs were washed, packed in containers and placed in an acaridarium to moult to the adult stage. The ensuing adult ticks (n=220) were used to infect Bovine 9446/6 with *T. parva*, which died from classical Corridor disease as confirmed by post-mortem examination, conventional PCR and RLB. A 10-fold dilution series of infected blood from buffalo KNP102 from 10^{0} to 10^{-6} was prepared in uninfected bovine blood. A blind experiment was performed: a set of thirty replicates of each dilution was made and the identity of the samples was concealed from the operator of the real-time PCR assay. DNA was extracted as previously described and eluted in 100 µl elution buffer. Two and a half microlitres of DNA were used in the amplification reaction with the *T. parva*-specific forward primer, *Theileria* genus-specific reverse primer and both probe sets. The estimated sensitivity and 95% confidence intervals for the true sensitivity for each group of dilutions prepared from KNP102 *T. parva*-infected blood were calculated using the standard error of the estimated sensitivity of each dilution group.

3.3.7 Comparison of the real-time PCR assay with other molecular tests

Three other molecular tests used for detection of *T. parva,* the conventional PCR/probe assay (PP) as described by Allsopp *et al*. (1993), the RLB (Gubbels *et al*., 1999) and the *cox*III PCR-based restriction fragment length polymorphism (RFLP) assay, described below, were selected for comparison with the real-time PCR assay. DNA samples from three gold standard positive animals (two cattle and one buffalo), 55 gold standard negative cattle and 309 field samples including both cattle and buffalo (Table 3.1) were subjected to the three tests and the real-time PCR assay using the *T. parva*-specific primer set. The sensitivity of the three tests in detecting *T. parva* was compared.

Cox*III PCR-based RFLP assay:* Primers were designed based on the *T. parva* cytochrome oxidase subunit III gene sequence (accession number: Z23263): Cox F [5'-CAA CAT TGT TAA AGC TAT CCA A-3'], Cox R [5'-ATG CGA AAC AGC GTA CAA TCA TA-3'] and Cox nR [5'-TTA TAG TAC AGG ATT AGA TAC CC-3']. A nested PCR was performed to amplify a *Theileria* genus-specific region in the *cox*III gene using primer sets Cox F and Cox R for the primary PCR and Cox F and Cox nR for the secondary PCR. The amplification mixture consisted of 1 µl yellow sub (GENEO BioProductions, Hamburg, Germany), 12.5 µl PCR buffer [KTT buffer: 150mM KCl, 30 mM Tris-HCl pH 8.6, 3% Triton X-100 and 3.3 mM MgCl2], 0.2 mM dNTPs, 0.4 µM of each primer, 0.5 U *Taq* polymerase and 5 µl DNA (~75 ng) in a final volume of 25 µl. Half a microlitre of the primary PCR product from a 25 µl PCR mixture was used as a template for the secondary PCR. A Hot start PCR programme was followed with the temperature of the thermocycler (BIOMETRA) (Whatman Biometra, Göttingeng, Germany) increased and held at 84°C until all the samples were loaded into the machine. For the primary PCR, the amplification programme included an initial denaturation step at 94°C for 4 min followed by 35 cycles of three steps each, comprising denaturing at 94°C for 45 sec, primer annealing at 59°C for 45 sec, and product extension at 72°C for 1 min. The PCR conditions for the secondary PCR were the same as described above except for the annealing temperature and amplification cycles which were reduced to 56°C and 25, respectively. The amplicons were digested with *Mbo*I overnight and separated on a 10% polyacrylamide gel before DNA detection by silver staining.

3.3.8 Proficiency testing

To determine the reproducibility of the real-time PCR assay, the test was performed in two different laboratories; at the Department of Veterinary Tropical Diseases (DVTD), Faculty of Veterinary Science, University of Pretoria and the Parasitology Division, ARC-OVI. A total of 115 blood samples (different from those used in the comparison of the real-time PCR assay with other molecular tests) including 20 cattle samples from known negative animals (vaccine animals), 41 buffalo samples from a *T. parva* endemic area, Hluhluwe-iMfolozi Game Reserve (expected to be positive) and 54 samples of unknown *T. parva* infection status, including cattle and buffalo received by the ARC-OVI for routine diagnostics, were used. A blind sample set was prepared and the DNA was extracted using the MagNA Pure LC System (Roche Diagnostics, Mannheim, Germany). The DNA samples were equally divided and subjected to the real-time PCR assay at the two laboratories. The data were analysed independently and forwarded to an independent analyst to determine the agreement of results.

3.4 Results

3.4.1 Specific detection of *T. parva* using the real-time PCR assay with hybridization probes

Initially, a primer set was designed for amplification of the V4 variable region of the 18S rRNA gene from all *Theileria* species. These *Theileria* genus-specific forward and reverse primers yielded a PCR product of approximately 230 bp from any *Theileria* species tested. The *T. parva*-specific hybridization probes were used for detection of *T. parva* amplicons generated using these primers. In *T. parva* positive samples, an increase in fluorescence was detected at 640 nm and melting curve analysis indicated that the *T. parva*-specific melting peak was at 63 ±0.62°C (Figure 3.2a). A smaller shoulder peak at 51 ±0.55°C was observed in all *T. parva* isolates. In addition to *T. parva* amplicons, the *T. parva*-specific hybridization probes recognised *T. taurotragi* and *T. annulata* PCR products generated by the *Theileria*-genus specific primers. As has been demonstrated even with single base differences in heterozygotes (Bollhalder *et al*., 1999), the nucleotide base differences in the amplicons of *T. taurotragi, T. annulata* and *T. parva* resulted in different melting peaks when used with the *T. parva*-specific hybridization probes, allowing discrimination between the different amplicons, with the *T. annulata* Tm at 48 ±0.09°C and *T. taurotragi* at 45 ±0.19°C (Figure 3.2b).

For the detection of the presence of any *Theileria* species in a sample, the *Theileria* genus-specific probe set was used to detect amplicons generated using the *Theileria* genus-specific primers. An increase in fluorescence at 705 nm was detected when all control *Theileria* DNA samples were tested, but it should be noted that this result gives no indication of which *Theileria* species is present.

To increase the specificity and sensitivity of the test, a *T. parva*-specific forward primer was designed for specific amplification of *T. parva* from a mixed infection, since competition between different templates could result in preferential amplification of the template with the highest starting concentration (Contamin *et al*., 1995). However, the *Theileria* sp. (buffalo) 18S rRNA sequence (accession number: DQ641260) is very similar to that of *T. parva* (accession number: L02366) and it was not possible to design an amplification primer that will not also amplify DNA from the former species. The *T. parva*-"specific" forward primer, used together with the *Theileria* genus-specific reverse primer, yielded a product of 167 bp

from both *T. parva* and *Theileria* sp. (buffalo) control samples. However, when these primers were used to amplify the *Theileria* control DNA samples in the presence of the *T. parva* probe set, a *T. parva*-specific melting peak at 63 ±0.62°C was only observed in the reaction containing *T. parva* positive control DNA (Figure 3.3). Except for *Theileria* sp. (buffalo) (see results below), no amplification or melting peaks at 640 nm were observed from any of the other *Theileria* species (Figure 3.3) or from any of the other blood parasites including *Babesia* spp., *Ehrlichia* spp., *Anaplasma* spp. and *Trypanosoma* spp. and bacterial DNA samples tested (Table 3.2). No increase in fluorescence in either the 640 nm or 705 nm channels was detected from any of the 55 negative bovine samples tested for *T. parva* (results not shown).

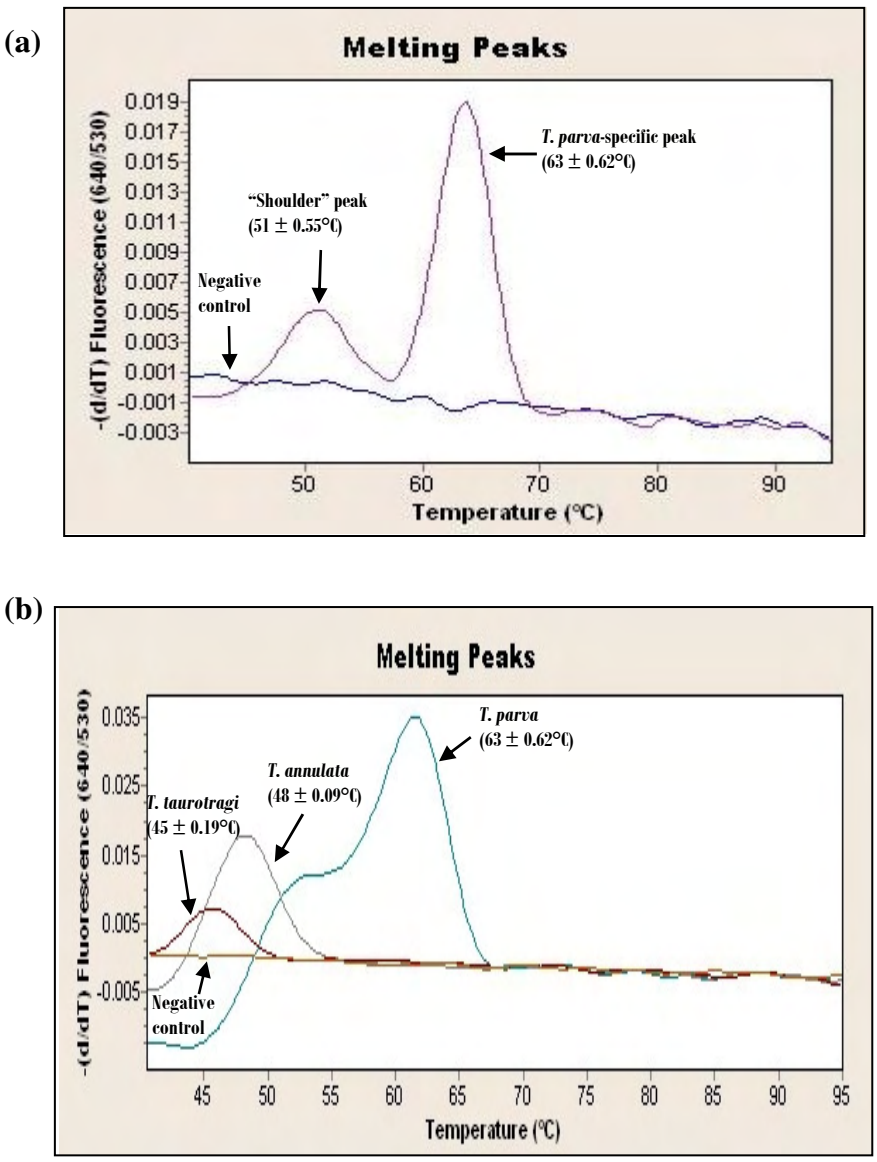

Figure 3.2 Detection of *T. parva* positive control DNA using the real-time PCR assay with *Theileria* genus-specific forward and reverse primers together with *T. parva*-specific hybridization probes. (a): Melting curve analysis showing *T. parva*-specific melting peak at 63 ±0.62°C and no fluorescence in the negative controls. (b): Discrimination between *T. parva, T. annulata* and *T. taurotragi* using melting curve analysis, with the melting peak for *T. parva* at 63 ±0.62°C, for *T. annulata* at 48 ±0.09°C and for *T. taurotragi* at 45 ±0.19°C.

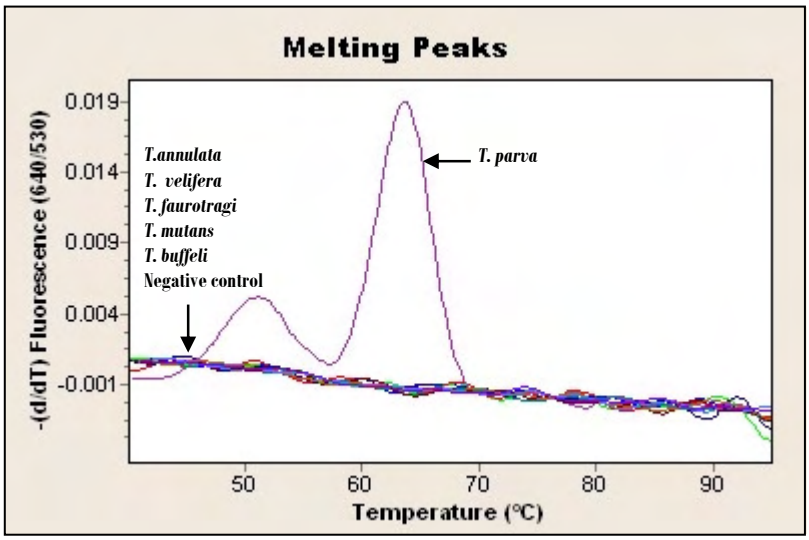

Figure 3.3 Specific detection of *T. parva* DNA. Amplicons were generated with the *T. parva*-specific primer together with the *Theileria* genus-specific reverse primer and detected with the *T. parva*-specific hybridization probe set. The *T. parva*-specific melting peak at 63 ±0.62°C was only observed in the *T. parva* positive control DNA samples, with no indication of amplification from any of the other *Theileria* species tested.

Theileria sp. (buffalo) control samples showed a slight increase in fluorescence at 640 nm (Figure 3.4a). An amplicon was obtained from *Theileria* sp. (buffalo) DNA as evidenced by an increase in fluorescence at 705 nm (Figure 3.4b), but no melting curve was produced in the 640 nm channel (Figure 3.4c). Therefore when both probe sets are included in a reaction with the *T. parva*-specific forward primer and the *Theileria* genus-specific reverse primer, an increase in fluorescence detected at 705 nm, but no melting curve at 640 nm indicates the presence of *Theileria* sp. (buffalo).

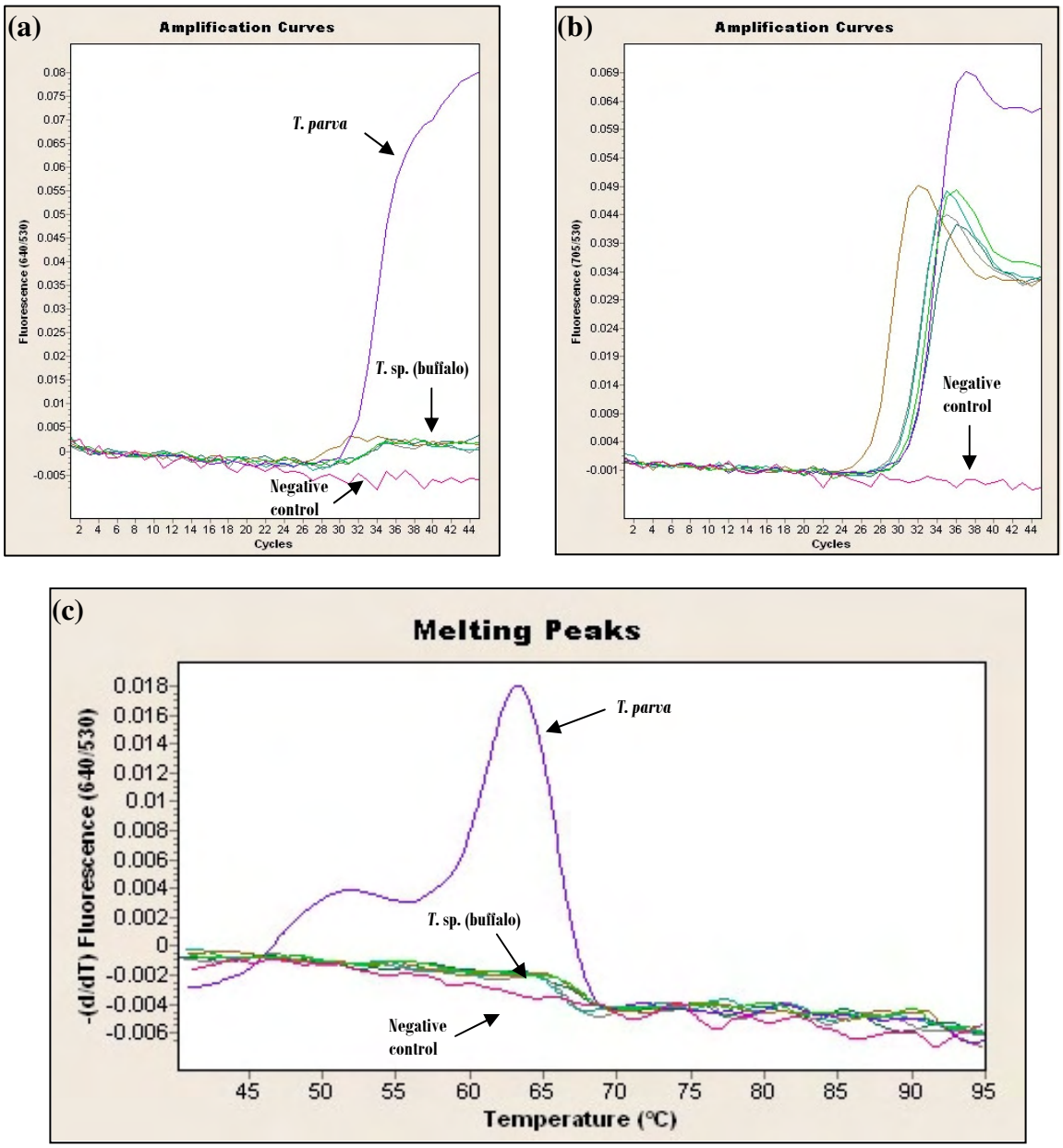

Figure 3.4 Discrimination between *T. parva* (blue lines), and *Theileria* sp. (buffalo) (green lines) using melting curve analysis. Amplicons were generated using the *T. parva*-specific forward primer and the *Theileria* genus-specific reverse primer, and detected with the *T. parva*-specific hybridization probe set. Amplification curves showing increase in fluorescence at (a) 640 and (b) 705 nm in *T. parva* and *Theileria* sp. (buffalo) DNA samples. (c): Melting curve analysis at 640 nm, showing the *T. parva*-specific melting peak at 63 ±0.62°C only for *T. parva* DNA.

3.4.2 Analytical sensitivity

From the set of dilutions prepared from *T. parva* positive buffalo KNP102, *T. parva* DNA was detected in all 30 replicates of the undiluted (3870 parasites/reaction) and the 10^{-1} dilution (387 parasites/reaction). As the dilutions increased, fewer of the 30 replicates tested positive until only one tested positive from each of the 10^{-5} (0.0387 parasites/reaction) and 10^{-6} (0.00387 parasites/reaction) dilutions (Figure 3.5).

At 3870 and 387 parasites/reaction (equivalent to $8.79x10^{-3}$ and $8.79x10^{-4}$% parasitaemia respectively) the sensitivity of the test was 100%; this decreased to 96.7% at 38.7 parasites/reaction ($8.79x10^{-5}$% parasitaemia) with a 95% confidence interval of 90.2%-100%. At 3.87 parasites/reaction ($8.79x10^{-6}$% parasitaemia) the probability of a positive test result, given that the individual tested actually has the parasite, was 80% with a 95% confidence interval of 65.7%-94.3%. The sensitivity of the test decreased to 26.67% at 0.387 parasites/reaction ($8.79x10^{-7}$% parasitaemia) with a 95% confidence interval of 10.8%-42.49% (Figure 3.5).

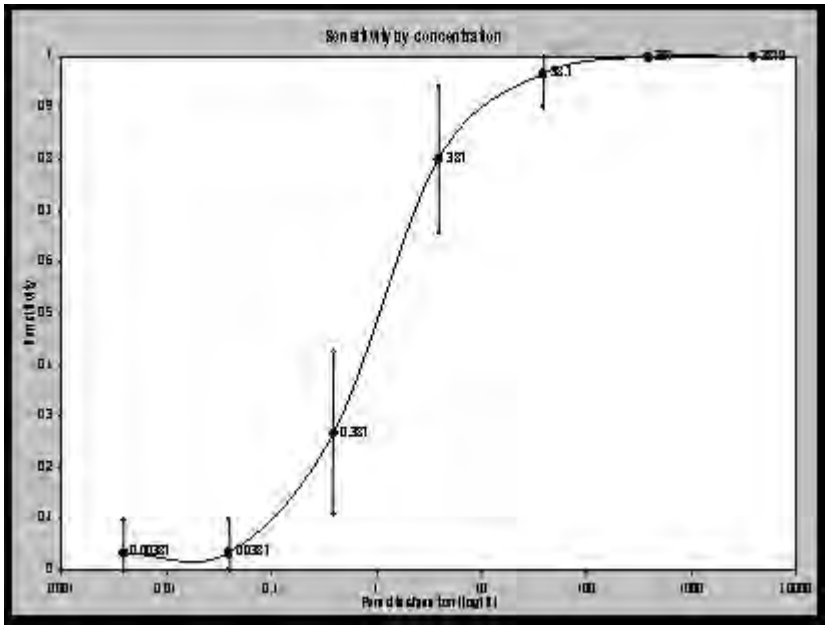

Figure 3.5 The sensitivity and 95% confidence intervals for a 10-fold dilution series from 10^{0} (3870 parasites/reaction) to 10^{-6} (0.00387 parasites/reaction) prepared from blood from a naturally infected buffalo (KNP102) with a parasitaemia of 0.009%.

3.4.3 Comparison of molecular tests

Three of the four molecular tests, namely the RLB, the real-time PCR and the *cox*III assays, detected *T. parva* DNA in all three gold standard positive samples, whereas the conventional PCR/probe assay detected *T. parva* DNA in only two of the three positive samples. *Theileria parva* was detected in 154 (49.8%) of the 309 field samples using the real-time PCR assay, 103 (33.3%) using the *cox*III assay, and 73 (23.6%) and 67 (21%) using the conventional PCR/probe assay and the RLB respectively (Figure 3.6a). All four assays detected *T. parva* DNA in the same samples. It should be noted that for the purposes of this experiment 1.0 µl of input DNA was used in the real-time PCR assay. Although the sensitivity of the real-time PCR assay was already better than the other tests using 1.0 µl of input DNA, it could be further improved by increasing the volume of input DNA. When input DNA was increased to 2.5 µl in 105 of the field samples, *T. parva* could be detected in 50 (52.4%) of the tested samples in contrast to 33 (34.3%) when 1.0 µl of input DNA was used (Figure 3.6b). Increasing the input DNA for the other three assays may have improved their sensitivities, but already their input DNA was relatively high [*cox*III assay (5 µl in a 25 µl reaction), conventional PCR/probe assay (2.5 µl in a 25 µl reaction), RLB (2.5 µl in a 25 µl reaction)] compared to that originally used for the real-time PCR assay (1.0 µl in a 20 µl reaction).

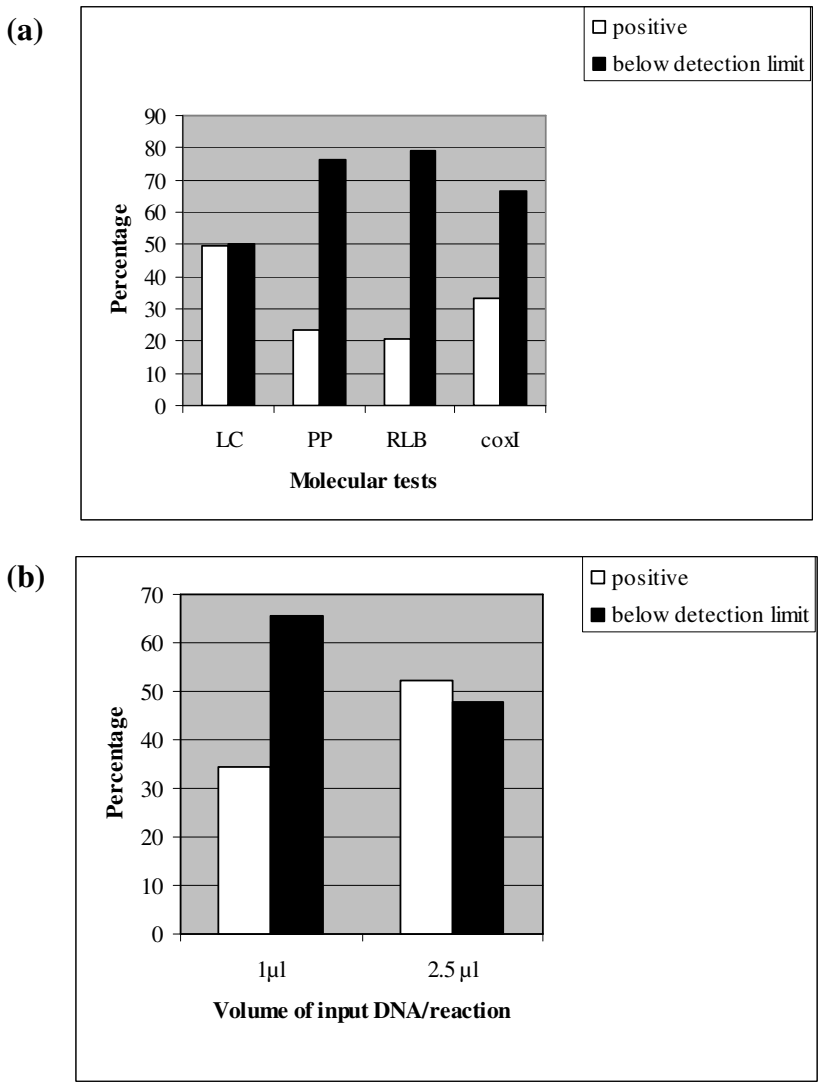

Figure 3.6 (a): Comparison of the sensitivity of the real-time PCR assay to that of other molecular assays in detecting *T. parva* from 309 field samples. One microlitre (~15 ng) of input DNA was used in the real-time PCR assay (LC), 2 µl (~30 ng) in the conventional PCR and probing test (PP), 2.5 µl (~37.5 ng) in the RLB, and 5 µl (~75 ng) in the *cox* III assay. (b): Improved ability of the real-time PCR assay to detect *T. parva* in 105 field samples when the input DNA was increased from 1 µl (~15 ng) to 2.5 µl (~ 37.5 ng)

3.4.4 Proficiency testing

A set of blood samples, including known negative animals, buffalo samples from a *T. parva* endemic area which were expected to be positive and diagnostic samples of unknown *T. parva* infection status, were subject to the real-time PCR test by different operators at the DVTD and OVI laboratories. Except for one sample, the laboratories obtained identical results which were also confirmed by an independent analyst (Figure 3.7).

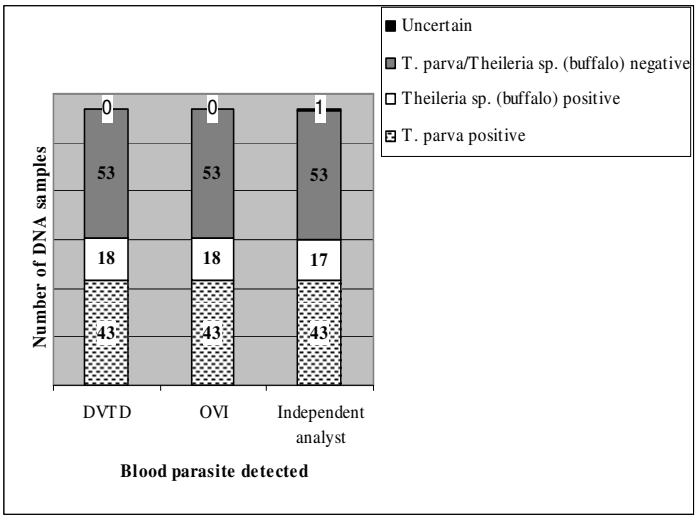

Figure 3.7 Comparison of results obtained from the DVTD and OVI laboratories when the real-time PCR assay was used to detect *T. parva*.

3.5 Discussion

A diagnostic assay for *T. parva* must be highly specific and sensitive in the presence of mixed infections, as the distribution of this pathogenic species coincides in many areas in southern Africa with that of other *Theileria* species, e.g. *T. mutans, T. velifera*, *T. buffeli*, *Theileria* sp. (buffalo) and *T. taurotragi* (Irvin, 1987; Norval *et al*., 1992). While the two sets of primers designed for the real-time PCR assay successfully amplified *T. parva* DNA under the conditions optimized for this assay, the *T. parva*-specific probe set also detected *T. taurotragi* and *T. annulata* when the *Theileria* genus-specific primers were used. Fortunately, this did not influence the specificity of the assay because the different products were easily discriminated by melting curve analysis.

The sensitivity of the real-time PCR assay may be compromised when *Theileria* genus-specific primers are used in samples containing mixed infections. In instances where *T. parva* infection is low and other *Theileria* species are present at higher levels, preliminary results indicate that competition for primers may result in a misdiagnosis of the species that is under-represented (data not shown). The specificity and sensitivity of the test were therefore improved by designing a *T. parva*-specific forward primer. However, since the *Theileria* sp. (buffalo) 18S rRNA gene sequence is very similar to that of *T. parva*, it was impossible to design an amplification primer that will not also amplify *Theileria* sp. (buffalo) DNA. Therefore, competition for primers between the different target sequences will still occur in mixed infections of *T. parva* and *Theileria* sp. (buffalo). Although an amplicon was generated from *Theileria* sp. (buffalo) DNA, the test still remained specific for *T. parva* since only a *T. parva*-specific melting curve was generated. It is possible that the *Theileria* sp. (buffalo) template-*T. parva* probe complex has a lower Tm than the starting temperature (40°C) used in the melting curve analysis. If this is the case, all *Theileria* sp. (buffalo) template-*T. parva* probe helices would have separated into single strands before the melting curve analysis began and no melting peak would have been observed. It is interesting that melting peaks were observed for *T. taurotragi* and *T. annulata* when the *Theileria*-genus specific primers were used. This may be explained by the fact that these sequences differ from the *T. parva* sensor probe sequence at two positions while there are three nucleotide differences in *Theileria* sp. (buffalo). Therefore the *T. taurotragi* and *T. annulata* template-probe complexes would have a slightly higher Tm and would still have been double-stranded at the beginning of the melt, explaining why melting curves were observed for these species but not for *Theileria* sp. (buffalo).

In addition to the peak at 63 ±0.62°C specific for *T. parva*, a shoulder peak was observed at 51 ±0.55°C. Such peaks can be due to mismatched bases in the probe region, but this was not the case in this study, since cloning and sequencing results from several *T. parva* isolates revealed no sequence variations between the two copies of the 18S rRNA gene. In addition, the same peak was observed when plasmids containing cloned *T. parva* 18S rRNA genes were subjected to the real-time PCR assay (results not shown). Such peaks are thought to result from back-folding of the amplicon on itself downstream of the sensor probe (Simpson *et al.*, 2007). This back-folding of the amplicon competes with the FRET probes binding to the amplicon and thus creates a lowered melting peak.

The real-time PCR test is extremely sensitive and can detect *T. parva* with 100% certainty in carrier animals with a piroplasm parasitaemia as low as $8.79x10^{-4}$%. However, in animals with lower parasitaemia, the test will be less reliable. It is not known whether a parasitaemia lower than $8.79x10^{-4}$% occurs in buffalo. In endemic areas where buffalo are constantly exposed to the parasite, the parasite load is likely to be within the detection limit of the real-time PCR test. However, in buffalo reared under tick-free conditions, the parasitaemia in infected animals, although fluctuating, is likely to remain extremely low, as observed in the naturally infected buffalo KNP102, since they are not exposed to constant re-infection. The ability of the real-time PCR assay to detect *T. parva* in such animals needs to be assessed.

The sensitivity of the real-time PCR assay was improved by increasing the amount of input DNA from 1.0 µl to 2.5 µl (~15 ng to ~37.5 ng) which increased the number of positive field samples by approximately 20%. Increasing the total volume of the real-time PCR reaction could further increase the sensitivity of the test. This would allow an even larger volume of input DNA, thereby increasing the chance of including parasite rDNA in the reaction. However, this would result in a fivefold increase in the cost of the test. Alternatively, DNA could be extracted from a larger volume of blood and eluted in a smaller volume, effectively concentrating the parasite DNA. Again this might increase the chance of including parasite rDNA in the reaction.

Several molecular tools have been developed for detection and differentiation of *Theileria* species (Morzaria *et al*., 1999). Most of these assays are based on conventional PCR and probing techniques (Bishop *et al*., 1992, Allsopp *et al*., 1993, Gubbels *et al*., 1999) and are relatively sensitive. However, they are laborious and time consuming. Real-time PCR tests have been developed for *Theileria* and *Babesia* parasites including *Theileria sergenti*, *Theileria equi, Babesia bovis and Babesia bigemina*, (Jeong *et al*. 2003; Kim *et al*., 2007, 2008). In most cases the sensitivity and specificity of real-time PCR tests not only compare well with those of conventional PCR-based methods, but significantly improve the sensitivity and specificity of the detection of these parasites, as in the case of the *T. parva* real-time test reported here. However, the *T. parva* real-time PCR test (based on hybridization probe chemistry) has an additional benefit over most real-time PCR tests developed for other *Theileria* and *Babesia* parasites (which are based on hydrolysis probe chemistry), as it is coupled with melting peak analysis, which confirms the identity of the amplified product.

Recently, loop-mediated isothermal amplification (LAMP) technology has been applied to the detection of *Theileria* and *Babesia* parasites (Iseki *et al*., 2007; Thekisoe *et al*., 2007). This technology allows amplification of as little as 1fg DNA in sixty minutes and is very cost effective as it does not require specialized equipment for amplification or analysis of amplicons. However, specific detection of *T. parva* has not yet been achieved using this technology and differentiation between *T. annulata*, *T. mutans*, *T. taurotragi* and *T. parva* amplicons is not possible (Thekisoe *et al*., 2007). In the case of mixed infections, a restriction enzyme analysis is required subsequent to amplification to allow differentiation of different parasite species, thus compromising the rapidity of the test (Iseki *et al*., 2007). The *T. parva* real-time PCR test reported here is currently the most rapid and reliable test available for specific detection of *T. parva* in cattle and buffalo in South Africa. This test is also more sensitive than other molecular assays currently used in the diagnosis of *T. parva* with its increased sensitivity accounted for by the fact that real-time PCR technology allows the measurement of the total amplification product in a reaction, in contrast to an aliquot that is analysed when using conventional PCR assays.

In May 2006, the newly developed real-time PCR test was adopted as a diagnostic test by the ARC-OVI, the only institution in South Africa authorized to test for *T. parva* infections in buffalo as part of the Corridor disease control strategy. To date, the assay has been used to test approximately 7420 field samples and 4% of these tested positive for *T. parva*. In the field, low piroplasm parasitaemias are a problem and continue to pose a challenge when interpreting the results. Melting curves are not well defined in some cases, which may be the result of low piroplasm parasitaemias and /or mixed infections with *Theileria* sp. (buffalo). Hence, buffalo from breeding projects are required to undergo five consecutive negative tests before they can be released on registered and approved properties only.

3.6 Summary

In summary, the real-time PCR assay reported here is specific for *T. parva* and more sensitive and faster than other molecular assays previously used in *T. parva* diagnostics. The assay is highly reproducible and has been shown to be reliable in the detection of *T. parva* piroplasm levels as low as $8.79x10^{-4}$%. However, sensitivity may still be a problem at infection levels lower than $8.79x10^{-4}$%.

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CHAPTER 4

Four p67 alleles identified in South African *Theileria parva* field samples

Trust in the LORD with all thine heart; and lean not unto thine own understanding. In all thy ways acknowledge Him, and He shall direct thy paths. ***Proverbs 3:5-6.***

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"I'm a slow walker, but I never walk back." ***Abraham Lincoln***
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4.1 Abstract

Previous studies characterizing the *Theileria parva* p67 gene in East Africa revealed two genotypes. Cattle-derived isolates associated with East Coast fever (ECF) have a 129 bp deletion in the central region of the p67 gene (allele 1), compared to buffalo-derived isolates with no deletion (allele 2). In South Africa, Corridor disease outbreaks occur if there is contact between infected buffalo and susceptible cattle in the presence of vector ticks. Although ECF was introduced into South Africa in the early 20th century, it has been eradicated and it is thought that there has been no cattle-to-cattle transmission of *T. parva* since. The variable region of the p67 gene was amplified and the gene sequences analyzed to characterize South African *T. parva* parasites that occur in buffalo, in cattle from farms where Corridor disease outbreaks were diagnosed and in experimentally infected cattle. Four p67 genotypes were identified, including alleles 1 and 2 previously detected in East African cattle and buffalo, respectively, as well as two novel genotypes, one with a different 174 bp deletion (allele 3), the other with a similar sequence to allele 3 but with no deletion (allele 4). Sequence variants of allele 1 were obtained from field samples originating from both cattle and buffalo. Allele 1 was also obtained from a bovine that tested *T. parva* positive from a farm near Ladysmith in the KwaZulu-Natal Province. East Coast fever was not diagnosed on this farm, but the p67 sequence was identical to that of *T. parva* Muguga, an isolate that causes ECF in Kenya. Variants of allele 2 were obtained from all *T. parva* samples from both buffalo and cattle, except Lad 10 and Zam 5. Phylogenetic analysis revealed that alleles 3 and 4 are monophyletic and diverged early from the other genotypes. These novel genotypes were not identified from South African field samples collected from cattle; however allele 3, with a p67 sequence identical to those obtained in South African field samples from buffalo, was obtained from a Zambian field isolate of a naturally infected bovine diagnosed with ECF. The p67 genetic profiles appear to be more complex than previously thought and cannot be used to distinguish between cattle- and buffalo-derived *T. parva* isolates in South Africa. The significance of the different p67 alleles, particularly the novel variants, in the epidemiology of theileriosis in South Africa still needs to be determined.

4.2 Introduction

Infections by *Theileria parva*, a tick-borne protozoan parasite, are responsible for classical East Coast fever (ECF), Corridor disease and January disease in cattle and normally inapparent infections in buffalo in eastern and southern Africa (Theiler, 1904; Neitz, 1955; Lawrence, 1992). The Cape buffalo (*Syncerus caffer*) is the natural reservoir host of *T. parva* and the parasite is transmitted by *Rhipicephalus appendiculatus, R. zambeziensis* and *R. duttoni* (Lawrence *et al*., 1983; Uilenberg, 1999).

East Coast fever was introduced into southern Africa in the early 1900s through the importation of cattle from East Africa, and was finally eradicated between 1946 and 1955 (Anonymous, 1981). In 1953, another form of cattle theileriosis, Corridor disease, was diagnosed after infected buffalo came into contact with cattle in the corridor of land between the then separate Hluhluwe and iMfolozi game reserves in South Africa (Neitz, 1955). The clinical symptoms and pathology of the disease were distinct from ECF. Schizont and piroplasm parasitoses were very low and it was thought that the parasite could not be transmitted between cattle, as affected animals usually died before piroplasms appeared. Corridor disease was thus considered to be caused by a different parasite, which was named *Theileria lawrencei* (Neitz, 1955). After the eradication of ECF in Zimbabwe, another form of theileriosis, known as January disease, emerged in that country, and the causative agent was named *Theileria bovis* (Lawrence, 1979; Uilenberg *et al*., 1982). Although the parasites causing ECF, Corridor Disease and January Disease were originally thought to be three different pathogenic species, they are now all considered to be *T. parva*, and *T. parva* isolates are now classified as cattle-derived or buffalo-derived (Perry and Young, 1993).

In southern Africa today, susceptible cattle sharing the same grazing as infected buffalo in the presence of vector ticks, can contract Corridor disease. The original buffalo-derived *T. parva* (the causative agent of Corridor disease) remains endemic in some parts of South Africa, hence the persistence of sporadic outbreaks of Corridor disease in South Africa. There is a concern that ECF could re-emerge in South Africa since cattle which recover from *T. parva* infections may become carriers of the parasite (Barnett and Brocklesby, 1966). A carrier state of ECF has also been shown to develop in cattle after immunization and treatment (Dolan *et al*., 1984; Maritim *et al*., 1989). Carrier state can develop in cattle that recover from Corridor disease following chemotherapy (Potgieter *et al*., 1985) and if this can also happen following natural infection, the disease may not be self-limiting as previously thought. Ticks

can be infected by feeding on carrier cattle and a situation may eventually develop where the parasite becomes adapted to cattle, resulting in cattle-to-cattle transmission. The South African cattle population would be highly susceptible should the parasite be introduced from an endemic area as the principal vector, *R. appendiculatus,* is still widespread. It is not known whether the parasite that caused ECF was transmitted to buffalo during the ECF epidemic, or whether South African strains of *T. parva* could eventually become adapted to cattle and cause ECF. There is therefore a need to establish the current status of *T. parva* parasites that are circulating in South African buffalo and possibly in cattle which may have recovered from buffalo-derived Corridor disease outbreaks.

In the past decade, several genes have been investigated in search of discriminatory sequence differences between *T. parva* isolates. Among these is the sporozoite antigen gene, p67 (Iams *et al*., 1990; Nene *et al*., 1996). Characterization of the p67 gene sequence in East Africa has revealed the presence of a 129 bp deletion in the central region in cattle-derived *T. parva* isolates, while there is no deletion in buffalo-derived isolates (Nene *et al*., 1992; 1996). Since the p67 sequences obtained from cattle-derived parasite stocks characterized in studies in East Africa were identical, it was assumed that the presence or the absence of the 129 bp deletion in the p67 gene could be used to differentiate between cattle- and buffalo-derived *T. parva* isolates (Nene *et al*., 1996). In South Africa, however, Collins (1997) obtained both p67 alleles in South African buffalo from the Kruger National Park. Although the p67 allele with a deletion was obtained from this isolate it was not established whether this particular strain could cause ECF.

The aim of this study was to characterize *T. parva* parasites that occur in buffalo and cattle in South Africa using sequence analysis of the p67 gene in an attempt to establish whether classical ECF-like parasites are present in South Africa.

4.3 Materials and methods

4.3.1 Sample collection

Cattle and buffalo blood samples were collected from different areas in South Africa. *Theileria parva* positive samples were selected using a *T. parva*-specific real-time PCR assay (Chapter 3; Sibeko *et al*., 2008). A total of 66 South African *T. parva* positive samples were characterized, including 62 field samples from cattle and buffalo and four experimentally

infected cattle (Table 4.1). One sample from a bovine (Zam 5) from Zambia was also analyzed.

4.3.2 DNA isolation

The High Pure PCR template preparation kit (Roche Diagnostics, Mannheim, Germany) was used to extract DNA from 200 µl of EDTA blood samples, according to the manufacturer's instructions. DNA was eluted in 100 µl elution buffer, rather than the recommended 200 µl, to increase the concentration of extracted DNA. The DNA was stored at 4°C until further analysis.

4.3.3 PCR amplification of the p67 gene from *T. parva*

Primers 613 (p67 forward primer) and 792 (p67 reverse primer) (Nene *et al*., 1996) were used to amplify the variable region of the p67 gene. Five microlitres of extracted DNA was used in a 25 µl PCR reaction and the amplification conditions applied were as described by Nene *et al*. (1996). For samples with low parasitaemia, 0.5 µl of the primary PCR product was used as a template for a secondary PCR using the same protocol but with the number of amplification cycles reduced from 40 to 25. The PCR products were analyzed by agarose gel electrophoresis.

Table 4.1 Source of samples used for characterization of *T. parva* parasites and results obtained from PCR amplification of the p67 gene. Table 4.1 continues in page 79.

Geographical origin of parasites	Province	Sample designation	Band size(s) obtained from PCR amplification (Kb)	Reference
Kruger National Park (KNP) (n=20)	Mpumalanga	KNP W8#	0.8, 0.9, 1.0, 1.1	
		KNP V5	0.9, 1.1	
		KNP 102#	0.8, 0.9, 1.0, 1.1	
		KNP 9446*#	0.9, 1.1	Sibeko *et al.* (2008)
		KNP B2	0.8, 0.9, 1.1	
		KNP M12	0.8, 0.9, 1.0, 1.1	
		KNP U3	0.9	
		KNP H12	1.1	
		KNP M2	0.8, 0.9, 1.0	
		KNP B1	1.0, 1.1	
		KNP B2	0.8, 1.1	
		KNP B15	0.9, 1.0, 1.1	
		KNP C5	0.9, 1.1	
		KNP O10	0.9, 1.0	
		KNP O11	1.1	
		KNP O14	1.0	
		KNP O17	0.8, 0.9, 1.0	
		KNP AA4	0.9, 1.0, 1.1	
		KNP A18	1.0	
		KNP A20	0.8, 0.9, 1.0, 1.1	
Ithala Game Reserve (n=9)	KwaZulu-Natal	Itha 2	0.8, 0.9, 1.0, 1.1	
		Itha 3#	0.8, 0.9, 1.0, 1.1	
		Itha 4	0.8, 0.9, 1.0, 1.1	
		Itha 5	0.8, 0.9, 1.0, 1.1	
		Itha 6	0.8, 0.9, 1.0, 1.1	
		Itha 7	0.8, 0.9, 1.0, 1.1	
		Itha 8#	0.8, 0.9, 1.0, 1.1	
		Itha 9	0.8, 0.9, 1.0, 1.1	
		Itha 10	0.8, 0.9, 1.0, 1.1	
Marakele National Park (n=11)	Limpopo	Mar 1.1	1.1	
		Mar 4	0.8, 0.9, 1.1	
		Mar 5	0.8, 0.9, 1.1	
		Mar 6	0.8, 0.9, 1.1	
		Mar 7	0.8, 0.9, 1.1	
		Mar 8	0.8, 0.9, 1.1	
		Mar 9	0.8, 0.9, 1.1	
		Mar 10	0.8, 0.9, 1.1	
		Mar 11	0.8, 0.9, 1.1	
		Mar 1#	0.8, 0.9, 1.1	
		Mar 75#	1.1	
Welgevonden Game Reserve (n=4)	Limpopo	Wel 23/04#	0.8, 0.9, 1.1	
		Wel 24/04#	0.8, 1.1	
		Wel 9288*#	1.1	Sibeko *et al.* (2008)
		Wel 9445*#	1.1	Sibeko *et al.* (2008)

Geographical origin of parasites	Province	Sample designation	Band size(s) obtained from PCR amplification (Kb)	Reference
Hluhluwe-iMfolozi Park (n=10)	KwaZulu-Natal	HIP 5#	0.8, 0.9, 1.0, 1.1	
		HIP 19	0.9	
		HIP 22	0.8, 0.9, 1.1	
		HIP 32	0.8, 0.9, 1.1	
		HIP 34	0.9	
		HIP 36	0.8, 0.9, 1.1	
		HIP 39	0.8, 0.9, 1.1	
		HIP 40	0.9	
		HIP 41	0.8, 0.9, 1.1	
		HIP 42	0.8, 0.9, 1.1	
Hluhluwe (n=1)	KwaZulu-Natal	**Hlu 9433*#**	1.1	Potgieter *et al.* (1988)
Ladysmith (n=4)	KwaZulu-Natal	**Lad 10#**	0.9	
		Lad 17#	1.1	
		Lad I238#	1.1	Thompson *et al.* (2008)
		Lad M119#	1.1	Thompson *et al.* (2008)
Bloemfontein (n=1)	Free-State	**Bloe B#**	1.1	
Mabalingwe Game Reserve (n=6)	Limpopo	Mab A13	0.9, 1.1	
		Mab B21	0.9, 1.1	
		Mab A22	0.9, 1.1	
		Mab BB37	0.9, 1.1	
		Mab BB38#	0.9, 1.1	
		Mab BB43#	1.0, 1.1	
Zambia (n=1)	East	**Zam 5#**	0.8	Geysen (2000)

All the samples in bold were obtained from cattle.
*Experimentally infected cattle.
PCR products obtained from these isolates were selected for sequencing.

4.3.4 Cloning and sequencing of p67 amplicons

Amplicons obtained from 21 selected samples (Table 4.1) were purified using the MinElute™ PCR Purification Kit (Qiagen, Venlo, the Netherlands). The p67 PCR products were cloned into the pGEM®-T Easy cloning vector (Promega, Madison, USA). Recombinant plasmid DNA was isolated using the High Pure Plasmid isolation kit (Roche Diagnostics, Mannheim, Germany). The presence of inserts was confirmed by colony PCR following the PCR protocol described by Nene *et al.* (1996). Three hundred to 450 ng of plasmid DNA was used in sequencing reactions prepared using the ABI Big Dye Terminator Cycle Sequencing kit version 3.1 (Applied Biosystems, Foster City, CA, USA). Sequencing was performed using a

SpectruMedix model SCE 2410 automated sequencer (SpectruMedix, State College, PA, USA) at INQABA Biotechnologies (South Africa).

4.3.5 Sequence analysis

4.3.5.1 Sequence editing

One hundred and forty sequences were obtained from clones produced from the 21 selected *T. parva* samples. Sequences were assembled and edited using GAP4 of the Staden package (version 1.6.0 for Windows) (Bonfield *et al*., 1995; Staden, 1996; Staden *et al*., 2000).

4.3.5.2 Sequence alignment

The p67 sequences obtained in this study were aligned with other published p67 sequences (Table 4.2). Sequences were initially aligned with the multiple sequence alignment program, MAFFT version 6 (Katoh *et al*., 2002) (http://align.bmr.kyushu-u.ac.jp/mafft/software/) and the final alignment was performed by eye using MacClade v4.0 (Maddison and Maddison, 1992). Alignment of nucleotide sequences was optimised using the amino acid reading frame. The lengths of the p67 sequence fragments varied between 751 bp and 967 bp. The first 167 bp and last 313 bp could be aligned without any difficulty. The middle region of the fragment was, however, highly divergent among strains and could not be reliably aligned across the entire data set. However, several strains shared similar insertions or deletions and these were coded as present or absent to represent a total of 13 unique synapomorphic characters. These characters can contribute phylogenetic signal, and therefore, to limit homoplasy, they were only scored if they comprised at least 9 base pairs in length (Matthee *et al*., 2001; 2007). The nucleotides comprising these inserts were deleted from further analyses.

Table 4.2 p67 reference sequences used for analysis of data obtained in this study

Geographical origin of parasites	Province	Isolate or sequence designation	Accession number	References
South Africa, Schoonspruit	Mpumalanga (former Transvaal)	Schoonspruit		Neitz (1948), Collins (1997)
South Africa, Kruger National Park (KNP)	Mpumalanga	KNP 1_S		Collins (1997)
		KNP 1_L1		Collins (1997)
		KNP 1_L2		Collins (1997)
		KNP 1_M		Collins (1997)
		KNP 2	AF079177	Nene *et al.* (1999)
South Africa, Hluhluwe	KwaZulu-Natal	Hluhluwe3	AF079176	Nene *et al.* (1999)
Uganda	North-west	Uganda		Minami *et al.* (1983), Morzaria *et al.* (1995), Collins (1997)
Kenya	Kilifi District	Muguga	M67476	Nene *et al.* (1996)
		Marikebuni		Irvin *et al.* (1983)
		7014	U40703	Nene *et al.* (1996)
		Waterbuck (Waterbuck experimentally infected with *T. parva* derived from buffalo 7014 which transmitted a subpopulation of parasites causing ECF in cattle.)		Stagg *et al.* (1994), Collins (1997)
		7344 (Tissue culture-clone generated from stock 7014.)		Morzaria *et al.* (1995), Collins (1997)

4.3.6 Phylogenetic analysis

Duplicate sequences were excluded from phylogenetic analyses and a total of 53 p67 sequences from nine representative samples were used. Parsimony analyses were performed in PAUP v4.0b10 (Swofford, 2003) based on heuristic searches with 100 random additions of taxa and tree bisection and reconnection (TBR) branch swapping. As a large number of equally parsimonious trees were found during each search the maximum number of trees saved during each replicate was constrained to 500. Data were analyzed using the 480 homologous nucleotides only and also in a combined fashion by adding the 13 unique characters that originated from length differences among the fragments. Substitutions were unordered and the 13 unique length differences among strains were analyzed unweighted (the insertion or deletion of each unique stretch of DNA contributed the same weight as a single nucleotide change), and weighted (3 times and 10 times heavier than a single nucleotide

change). One thousand parsimony bootstrap replicates were performed to obtain confidence values for the nodes.

4.4 Results

4.4.1 Amplicon analysis by agarose gel electrophoresis

Two p67 alleles have previously been reported in East Africa, the p67 gene with a 129 bp deletion (designated allele 1 in this study) and the p67 gene with no deletion (designated allele 2) (Nene *et al*., 1996). In this study, up to four p67 PCR products of sizes ~ 0.8, 0.9, 1.0 and 1.1 kb, were obtained (Figure 4.1 and Table 4.1). It is more than likely that the multiple bands obtained in this study are authentic and representative of several different *T. parva* parasites present in a single sample, because p67 is a single copy gene.

A single amplicon was obtained from all *T. parva* positive samples obtained from cattle, from both experimentally infected and field samples, except for one, bovine KNP 9446, from which a double band was obtained (Table 4.1). The PCR product sizes obtained from KNP 9446 consisted of the 1.1 and 0.9 kb fragments. A 0.9 kb PCR product was obtained from Lad 10, a 0.8 kb PCR product from Zam 5 and 1.1 kb amplicons were obtained from all other cattle samples.

Single amplicons of band sizes 0.9, 1.0 or 1.1 kb, were obtained from 10 buffalo samples; otherwise, two to four amplicons were obtained from *T. parva* field samples originating from buffalo. The 0.8, 0.9, 1.0 and 1.1 kb bands were present in, respectively, 61%, 77%, 39% and 82% of the 57 buffalo samples analyzed.

Heterogeneous p67 PCR product profiles were obtained from *T. parva* samples from buffalo from the Kruger National Park and Hluhluwe-iMfolozi, while the p67 PCR products obtained from Ithala, Marakele and Mabalingwe samples appeared to be homogeneous within each game reserve. PCR products of the same sizes were obtained from *T. parva* samples from buffalo from Ithala (with 100% of samples producing all four PCR products), Marakele (with 81 % of samples producing the 0.8, 0.9 and 1.1 kb PCR products) and Mabalingwe (with 83% of samples producing the 0.9 and 1.1 kb PCR products).

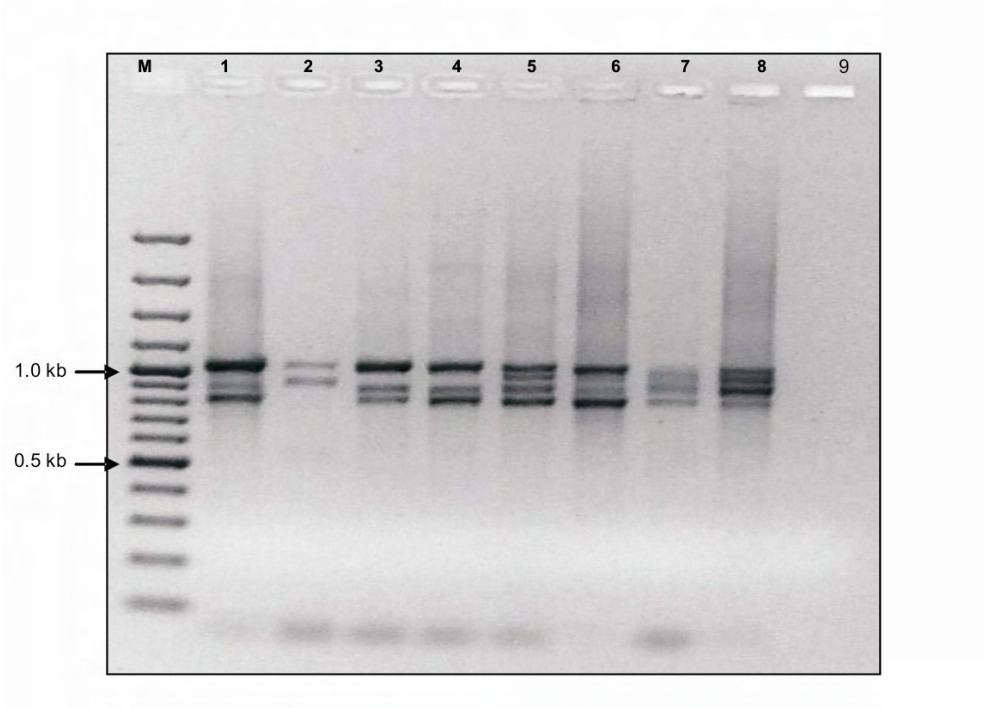

Figure 4.1 Amplicon profiles obtained from amplification of the central region of the p67 gene from buffalo-derived *T. parva* isolates collected from different geographical areas in South Africa. Lanes: M=1 kb plus DNA marker (Fermentas Life Sciences), 1= Wel 23/04, 2= Mab 43, 3=Mar 1, 4= Itha 3, 5= Itha 8, 6= HIP 5, 7 = KNP W8, 8= KNP 102, 9 = negative control. See Table 4.1 for geographical origin of isolates.

4.4.2 Sequence analysis

The p67 sequences obtained in this study were aligned with the published sequences shown in Table 4.2. Four groups of p67 sequences were identified (Figure 4.2), including the previously identified alleles 1 and 2, as well as two novel alleles, one with a different 174 bp deletion (allele 3), the other with no deletion (allele 4). The deletion in allele 3 occurs ~ 20 bp upstream of the position where the deletion occurs in allele 1 (Figure 4.2).

Sequences characteristic of alleles 1 and 2 were obtained from the 0.9 and 1.1 kb amplicons, respectively, and allele 3 and 4 sequences were obtained from the 0.8 and 1.0 kb PCR products, respectively (Table 4.3). Although it was possible to sequence all bands from all samples, at least two p67 allele sequences were obtained from *T. parva* samples originating from buffalo (Table 4.3), except for Mar 75 which had one p67 allele. Sequences of all four alleles were present in two individual isolates, KNP W8 and KNP 102, and three in Itha 8 and

HIP 5. Allele 2 sequences were obtained from all *T. parva* samples originating from cattle except two, Lad 10 and Zam 5 (Table 4.3), which had alleles 1 and 3, respectively. In addition to allele 2 sequences, KNP 9446, a bovine experimentally infected with *T. parva* parasites from buffalo KNP102 (Chapter 3; Sibeko *et al*., 2008) also contained parasites with p67 allele 1 sequences. Allele 1 was also obtained from a naturally infected *T. parva*-carrier bovine, Lad 10, originating from a farm near Ladysmith, and the sequence was identical to that of *T. parva* Muguga. An allele 3 p67 sequence, similar to allele 3 sequences obtained from South African *T. parva* field samples originating from buffalo, was obtained from a Zambian isolate, Zam5, originating from a naturally infected bovine. Sequence variants of alleles 1 and 2, similar to p67 sequences from a sample obtained from a buffalo at KNP in 1994 reported by Collins (1997), namely KNP1_M and KNP1_L1, were also obtained. These p67 variants had sequences similar but not identical to previously reported alleles 1 and 2 (Nene *et al*., 1996). The novel sequences, alleles 3 and 4, obtained in this study were similar to KNP1_S and KNP1_L2 p67 sequences, respectively, reported by Collins (1997). Among allele 2 sequences, sequence variations within the 129 bp region specific to buffalo-derived parasites were observed (Figure 4.2).

Cloning and sequence analysis also revealed the presence of more than one p67 sequence variant from what appeared to be a single PCR product (or single band on an agarose gel). For example, two different sequence variants of allele 2 were obtained from DNA clones prepared from the amplicons obtained from experimentally infected bovine, Hlu 9433, and a naturally infected bovine, Lad M119 (results not shown).

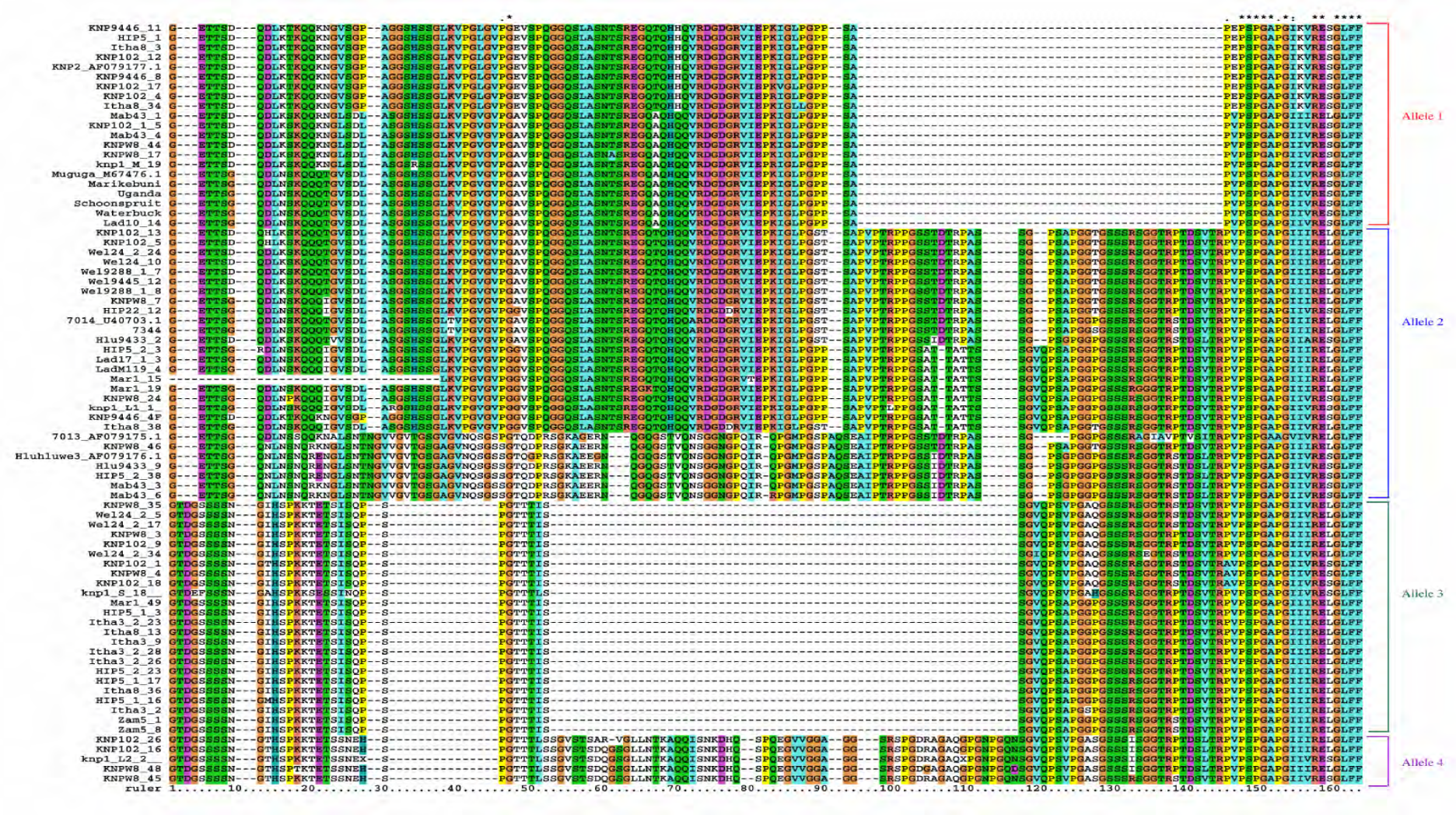

Figure 4.2 Alignment of the inferred amino acid acid sequences of a portion of the ~600 bp variable region of the p67 gene amplified from representative *T. parva* strains. The alignment was generated using the multiple sequence alignment program Mafft version 6 (Katoh *et al*., 2002) (**http://align.bmr.kyushu-u.ac.jp/mafft/software/**).

Table 4.3 Number and type of p67 sequences obtained from 21 selected *T. parva* samples collected from both cattle and buffalo

Sample name	Total number of clones sequenced [140]	Number of p67 sequences			
		Allele 1 (0.9 kb)	Allele 2 (1.1 kb)	Allele 3 (0.8 kb)	Allele 4 (1.0 kb)
KNP W8	10	2	3	3	2
KNP 102	16	6	4	3	3
Itha 3#	8	-	3	5	-
Itha 8#	16	7	4	5	-
HIP 5#	15	6	3	6	-
Mab BB38	6	3	3	-	-
Mab BB43	9	6	3	-	-
Wel 23/04#	3	-	3	-	-
Wel 24/04	9	-	5	4	-
Mar 1#	11	-	6	5	-
Mar 75	3	-	3	-	-
KNP 9446*	4	2	2	-	-
Zam 5	2	-	-	2	-
Wel 9288*	6	-	6	-	-
Wel 9445*	2	-	2	-	-
Hlu 9433*	3	-	3	-	-
Lad 17	5	-	5	-	-
Lad M119	4	-	4	-	-
Lad I438	4	-	4	-	-
Lad 10	1	1	-	-	-
Bloe B	3	-	3	-	-

All the samples in bold were obtained from cattle.
Note that not all bands were sequenced from these samples.
*Experimentally infected cattle.

4.4.3 Phylogenetic analysis

Analyses of the 480 flanking nucleotides (60 parsimony informative characters) resulted in more than 500 equally parsimonious trees which were largely unresolved after bootstrap analyses (<50%). Although 13 nodes were supported by ≥50% bootstrap support these were mostly restricted to the terminal associations among strains. Inclusion of the 13 unique insertions or deletions significantly increased the phylogenetic resolution and bootstrap support was obtained for an additional 15 nodes when the analyses included 73 parsimony informative characters (60 nucleotides and 13 insertions/deletions). The majority of the additional signal resolved the more basal associations in the topology (among alleles).

Two major clades, A and B, were identified from the most parsimonious tree (Figure 4.3). Alleles 3 and 4 clustered together in a single clade (A) suggesting that they diverged early and evolved separately from other p67 alleles. Clade B included known alleles 1 and 2 and their sequence variants. Each major clade consisted of subgroups that could also be divided based on the presence or absence of the deletion (Figure 4.3). Subgroup A2 consisted of novel p67 sequences with a deletion (allele 3) and A1 consisted of novel sequences with no deletion (allele 4). Similarly in clade B, subgroup B1 consisted of p67 sequences with a different deletion (allele 1) while B2 consisted of sequences without the deletion (allele 2). B2 formed a basal clade for B1 suggesting that allele 1 is derived from allele 2. The Muguga p67 sequence together with other cattle-derived isolates shared a common ancestor, clade C, with groups D1, D2 and E comprising p67 allele 1 variants. Cattle-derived sequences [Muguga, Schoonspruit, Marikebuni, *T. parva* (waterbuck-passaged) and Uganda (Table 4.2)] and sequence variants grouping with the KNP2 p67 sequence (Table 4.2) in subgroup B1 appeared to be monophyletic. p67 sequences from a Zambian isolate obtained from a naturally infected bovine diagnosed with ECF (Geysen, 2000) grouped with South African novel allele 3 sequences, in clade A subgroup A2.

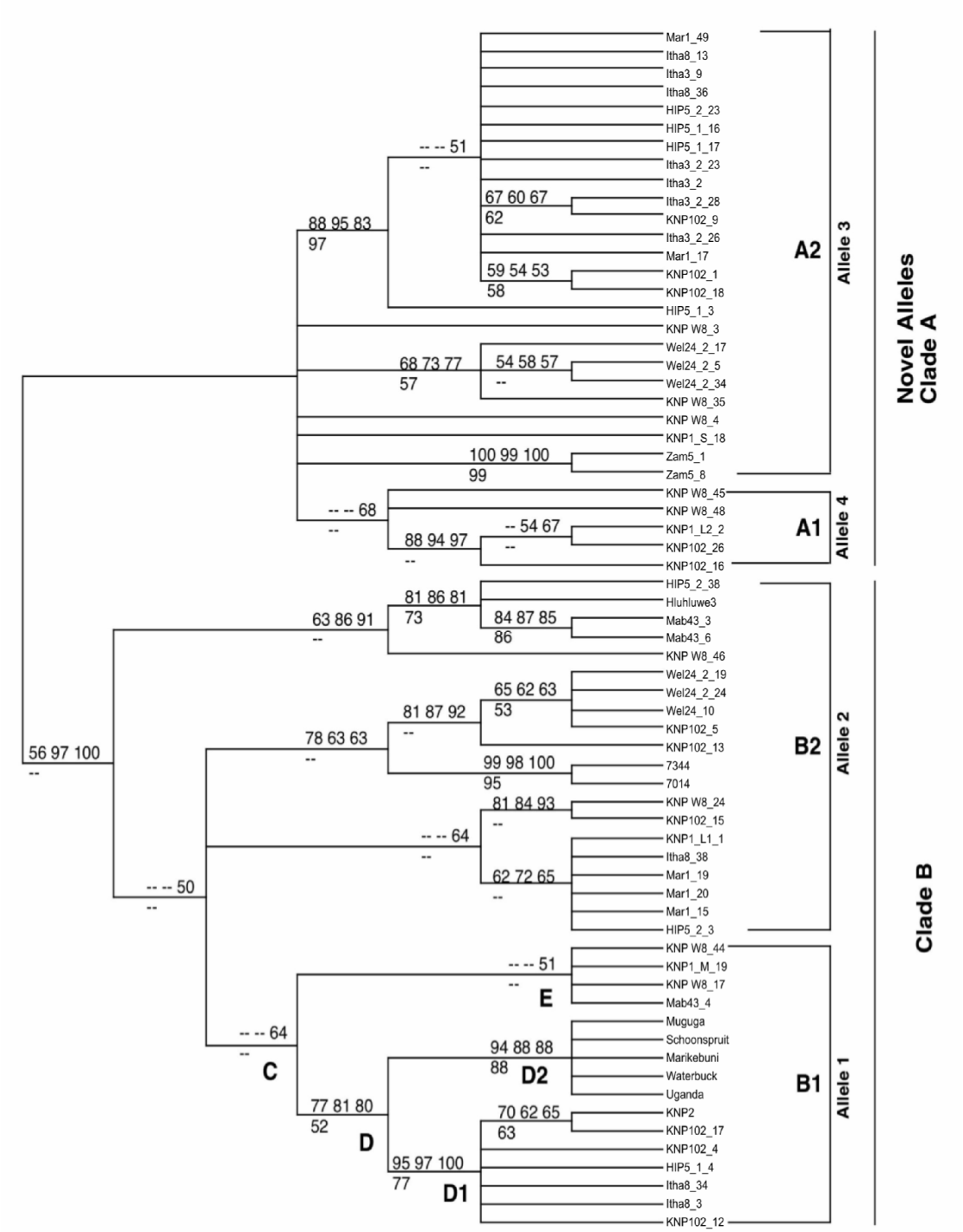

Figure 4.3 Phylogenetic relationship of *T. parva* strains as revealed by p67 gene sequences. The phylogenetic tree was calculated by maximum parsimony analysis using TBR swapping in PAUP*4.0b10 (Swofford, 2003) and the tree where unique insertions are weighted 10:1 is shown. Nodal support was assessed with 1000 bootstrap replicates and indicated above for 1:1; 1:3; 1:10 weighting of indels while values below represent bootstrap support for the nucleotide analyses only (see text for details).

4.5 Discussion

According to data obtained in East Africa, the cattle-derived *T. parva* isolates, Boleni, Muguga, Marikebuni, Mariakana and Uganda, have an identical p67 gene sequence which contains a 129 bp deletion (allele 1) and this deletion is not present in the p67 gene sequence from buffalo-derived isolates (allele 2) (Nene *et al.*, 1996). Consequently, it has been speculated that all *T. parva* stocks which can be maintained by passage between cattle and the tick vector, have the same p67 gene sequence containing the 129 bp deletion (Nene *et al.*, 1996). In this study both alleles were obtained from many of the *T. parva* field samples obtained from buffalo and cattle. In addition to alleles 1 and 2, two novel alleles (alleles 3 and 4) were also identified by PCR and sequence analysis. Although it is possible that PCR artifacts, such as overlap extension, could result in sequence variants, there was no evidence in the data obtained in this study to suggest that overlap extension may have occurred. A non-PCR based method such as Southern blot could have been used to confirm that the variants characterized by deletions were authentic; however, the parasite DNA in field samples is often too low to detect even by PCR. The sporozoite antigen gene, p67, codes for a stage-specific protein involved in the process of entry of the *T. parva* sporozoite into the host lymphocytes (Webster *et al.*, 1985; Shaw, 2003). It is possible that the p67 allele type might be associated with the ability of the parasite to infect a specific host, which could explain the apparent selection of parasites with p67 allele 1 in cattle in East Africa.

Analysis of p67 PCR product profiles indicated that four p67 alleles are present in *T. parva* parasites in buffalo in South Africa. Alleles 1 and 2 occurred more frequently than alleles 3 and 4. Relatively uniform p67 profiles were obtained from *T. parva* samples from buffalo from Ithala, Marakele and Mabalingwe game reserves, suggesting that homogeneous populations of parasites could be circulating among buffalo on these properties. On the contrary, more heterogeneous p67 PCR product profiles were obtained from samples from Kruger National Park and Hluhluwe-iMfolozi Park suggesting an extensive diversity of the parasite population occurring in larger populations of buffalo.

In this study, a 0.9 kb PCR product, representative of p67 allele 1, was found in 77% of the *T. parva* samples obtained from buffalo. All of the p67 allele 1 sequences from samples from buffalo were variants of the previously reported allele 1 from isolates obtained from cattle, with a number of amino acid substitutions which distinguished them from the known cattle-derived p67 allele 1. One such variant has previously been obtained from KNP2, a stock

originating from a naturally infected buffalo cow captured in the southern part of the Kruger National Park (Collins, 1997; Nene *et al*., 1999). *Rhipicephalus zambeziensis* nymphs were fed on this buffalo, and adult ticks reared from these nymphs transmitted Corridor disease to an adult bovine cow, B9678-2. Schizont-infected lymphoblastoid cells were established *in vitro* from lymph node aspirates obtained from this animal (H. Stoltsz, pers. comm.). It is possible that this cell culture is representative of a sub-population of the *T. parva* parasites that were present in the original buffalo from which the *T. parva* KNP2 isolate was prepared. Selection of a subpopulation of parasites may have occurred in the *T. parva* KNP2 stock, either *in vivo* or in cell culture. However, evidence is required to establish if this *T. parva* stock can be maintained in cattle. In a separate tick transmission study, parasites with a similar variant of allele 1 and also allele 2 were transmitted from buffalo KNP 102, which had a multiple infection of *T. parva* parasites possessing all four p67 alleles, into bovine KNP 9446/6. However, this animal died from classical Corridor disease (Chapter 3; Sibeko *et al*., 2008).

Only one p67 sequence obtained from a naturally infected bovine, Lad 10, from a farm near Ladysmith, KwaZulu-Natal, was identical to the typical cattle-derived p67 sequence, allele 1 (Nene *et al*., 1999). It is not known if the parasites that infected bovine Lad 10 originated from buffalo or cattle; however, this animal did not exhibit any disease symptoms associated with *T. parva* infection, suggesting that it was a carrier.

Except for Lad 10, all p67 gene sequences obtained from samples originating from cattle from the Ladysmith farm were typical of buffalo-derived *T. parva* parasites (allele 2). In fact, three of the Ladysmith samples (Lad 17, Lad I438 and Lad M119) had p67 sequences that were similar to sequences identified in *T. parva* field samples HIP 5, HIP 32 and HIP 39, obtained from buffalo from Hluhluwe-iMfolozi, a game park in KwaZulu-Natal. While this suggests that the *T. parva* parasites present in cattle on this farm could have been derived from buffalo, it is not clear if these cattle had any contact with infected buffalo (Thompson *et al*., 2008).

Together, these results indicate that the additional determinants which result in ECF, including the ability to produce microschizonts and high piroplasm parasitaemias, were probably not present in the parasites sampled in this study. It is not known what these determinants are or whether they occur in *T. parva* parasites in South Africa. However, it is apparent from these findings that *T. parva*-carrier cattle containing parasites possessing p67 allele 1 are present, at least on one farm, in South Africa. This finding is of concern to the

cattle industry in South Africa, since Potgieter *et al.* (1988) showed that buffalo-derived *T. parva* parasites causing Corridor disease can be maintained by passage between cattle and the tick vector. The persistence of *T. parva*-carrier cattle in South Africa could eventually result in the selection of *T. parva* parasites adapted to cattle.

In addition to p67 alleles 1 and 2, two novel variants were obtained, allele 3, with a deletion and allele 4, with no deletion. Allele 3 was obtained from samples originating from both buffalo and cattle (Zam 5) whereas allele 4 was only obtained from *T. parva* field samples originating from naturally infected buffalo. Zam 5 is an isolate obtained from a naturally infected bovine diagnosed with ECF symptoms in the Southern Province of Zambia (Geysen, 2000). The p67 sequence obtained from this isolate was identical to the South African allele 3 sequences. This result suggests that *T. parva* parasites carrying the novel allele 3 can be transmitted to cattle and indicates that parasites that cause ECF do not exclusively contain p67 allele 1.

A majority of *T. parva* field samples from which novel p67 variants were obtained also contained *T. parva* parasites with p67 alleles 1 and 2. This was not surprising as it is expected that buffalo, as reservoir hosts, will harbour more *T. parva* strains than exist in cattle as a result of recombination occurring in the tick vector. The phylogenetic analysis presented here indicates that parasites containing p67 alleles 3 and 4 seem to have evolved separately from cattle- and buffalo-derived parasites carrying p67 alleles 1 and 2, which is surprising, given the extensive recombination known to occur between *T. parva* parasites in the tick vector (Nene *et al.*, 1998). It is possible that these parasites were introduced during the ECF epidemic along with ECF-causing parasites from East Africa, but from the results obtained in this study, it is not possible to tell whether this is the case or whether these parasites have always existed in buffalo. In addition, there are no reports of *T. parva* parasites with novel p67 alleles (alleles 3 and 4) in other East African countries such as Tanzania or Kenya. However, it is very likely that these parasites also occur there given that there was historically great connectivity between buffalo populations in East and southern Africa (Van Hooft *et al.*, 2000), and it has been shown in this study that these novel variants are also present in Zambia. In the same manner that ECF was introduced into South Africa, ECF was introduced to the Northern Province of Zambia by importation of cattle from Tanzania in 1922 (Nambota *et al.*, 1995). The disease spread to Southern Province in the early 1970s. Interestingly, the novel variant was obtained in the Southern Province from an animal with ECF (Geysen, 2000). It is of interest that it was obtained from an area known to be frequented by buffalo. If novel p67

variants do exist in countries in East Africa, it will be interesting to establish whether parasites characterized by the novel p67 alleles are implicated in ECF cases there.

Sequence analysis revealed that there are more *T. parva* p67 alleles in South African buffalo than have previously been recognized. This confirms the extensive diversity in buffalo-derived *T. parva* parasites that has previously been reported (Conrad *et al*., 1987; 1989; Morzaria *et al*., 1995; Nene *et al*., 1996; Collins and Allsopp, 1999). Not only were alleles 1 and 2 identified in this study, but also many variants of these sequences. Variants of p67 allele 1 were obtained from some *T. parva* field samples originating from buffalo; from phylogenetic analysis, these sequences group together with the Muguga p67 sequence (allele 1) in clade C. The phylogenetic analysis further suggests that allele 1 associated with ECF (clade D2) is closely related to variants of allele 1 in clades D1 and E from field samples originating from South African buffalo. As indicated earlier, parasites that occur in the South African buffalo population probably occur in the East African buffalo population as a result of historical buffalo migration; it will therefore be interesting to establish whether parasites in clades D1 and E are implicated in ECF cases in East Africa and if so, why this is not the case in South Africa. The phylogenetic analysis presented here suggests that the cattle-derived p67 *T. parva* alleles evolved from buffalo-derived p67 alleles, supporting the belief that *T. parva* is originally a buffalo parasite (Uilenberg, 1981; Young, 1981; Norval *et al*., 1992) and the hypothesis that selection of a subpopulation of *T. parva* parasites resulted in ECF (Young, 1981; Conrad *et al*., 1989).

In South Africa, cattle are kept separate from buffalo to prevent infection of susceptible cattle. Recently cattle have been observed grazing around the borders of game reserves even in Corridor disease endemic areas. This situation might result in transmission of the parasite from infected buffalo to susceptible cattle and could result in the circulation of the parasite in the cattle population. As a result, there would be a higher risk of genetic exchange (Nene *et al*., 1998) that might eventually result in a parasite population that could cause ECF. It should be noted, however, that in South Africa, attempts to demonstrate transformation of buffalo-derived *T. parva* parasites to the cattle-type have proven futile (Neitz, 1957; Potgieter *et al*., 1988) although the same experiments in East Africa were successful (Barnett and Brocklesby, 1966; 1969; Young and Purnell, 1973; Maritim *et al*., 1992). Nevertheless, should transformation or DNA recombination occur, resulting in emergence of a parasite population that can cause ECF, the cattle population in South Africa would be vulnerable. Therefore it is imperative that markers are identified which can be directly linked to the

disease syndrome of *T. parva* parasites in order to provide informative molecular epidemiological data which might help the South African veterinary authorities to make informed decisions in the control of theileriosis.

4.6 Conclusion

Theileria parva p67 gene profiles appear to be more complex than previously thought. It is apparent from the results obtained in this study that the typical buffalo- and cattle-derived p67 profile as established in East Africa cannot be used to distinguish between cattle- and buffalo-derived *T. parva* parasites in South Africa and that parasites with p67 genes that have the 129 bp deletion (allele 1), as in cattle-derived isolates, cannot be associated with a specific disease syndrome. Therefore, it is still necessary to identify markers which could be directly associated with the different disease syndromes. The significance of the different p67 alleles, particularly the novel variants, in the epidemiology of theileriosis in South Africa still needs to be determined.

4.7 References

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