CHAPTER 7

Evaluation of a "pan" FRET real-time PCR test for the discrimination of *Theileria* species in the African buffalo (*Syncerus caffer*)

7.1 Abstract

A quantitative fluorescence resonance energy transfer (FRET)-real-time PCR (qPCR) assay based on the cox III gene was developed for the simultaneous detection and discrimination of *Theileria* species in cattle using melting curve analysis. The cox III qPCR assay can detect *Theileria parva*, *Theileria mutans*, *Theileria velifera*, *Theileria annulata*, *Theileria taurotragi* and *Theileria buffeli* in infected cattle using a nested qPCR assay with a single set of probes. The assay was modified, by the development of a new set of primers and probes, to include the detection of *Theileria* sp. (buffalo) from buffalo samples. In the current study, we evaluated the modified cox III qPCR assay for the identification and discrimination of *Theileria* spp. of the African buffalo.

The results of the cox III qPCR assay were compared with the reverse line blot (RLB) hybridization assay for the simultaneous detection and differentiation of *Theileria* spp. in 220 buffalo and 4 cattle blood samples from South Africa and Mozambique. The following species were detected using the cox III qPCR assay: *T. parva* (83.5%), *Theileria* sp. (buffalo) (55.8%); *T. taurotragi* (1.8%), *T. buffeli* (5.8%) and *T. mutans* (2.2%). Seventeen percent of the samples had non-specific melting peaks and 4.5% of the samples were negative or below the detection limit of the assay. *Theileria velifera* was not detected from any of the samples analysed by the cox III qPCR. The assay detected more *T. parva* and *Theileria* sp. (buffalo) infections but fewer *T. mutans* and *T. buffeli* infections than the RLB assay.The cox III qPCR test identified *Theileria* spp. infections in samples that were negative or below detection limit of the RLB assay and therefore seems to be the more sensitive test. However, the identification of *T. taurotragi* by the cox III qPCR assay in some buffalo samples was unexpected. To our knowledge *T. taurotragi* has never been isolated from the African buffalo. Because of the discrepancies between *Theileria* species identified by the RLB and cox III qPCR assays, the identification of *T. taurotragi* in buffalo samples, and the non-specific peaks observed in the cox III qPCR assay, cox III qPCR products were cloned and the resulting clones were

sequenced. Sequencing and phylogenetic analysis indicated extensive sequence differences, in both the number of genotypes and co-infecting genotypes, in buffalo and explained the apparently low occurrence of benign *Theileria* spp. as detected by the cox III qPCR assay. The cox III qPCR was subsequently also compared with the 18S qPCR assay for the specific detection of *T. parva* in 206 samples. *Theileria parva* was detected in 51.9%, 79.2% and 86.4% of the 206 samples analysed by the RLB, 18S qPCR and cox III qPCR assays, respectively

7.2 Introduction

Theileria parva is the causative agent of Corridor disease (theileriosis) in cattle in South Africa and the African buffalo (*Syncerus caffer*) is the reservoir host. As theileriosis and other diseases carried by buffalo are a threat to farming communities in the endemic areas of the country (Uilenberg, 1999), interaction between cattle and buffalo is limited. Buffalo must test negative for *T. parva* before translocation, and this has resulted in an increased demand and cost of disease-free animals (Collins et al., 2002). The tests used for the diagnosis of *T. parva* should therefore be sensitive and specific for accurate diagnosis. However, benign and non-pathogenic *Theileria* species that co-exist with *T. parva* in infected animals can compromise the specificity and sensitivity of the tests used.

Polymerase chain reaction (PCR) assays are more sensitive and specific than microscopy and serological methods, and usually limit the subjectivity that occurs in the interpretation of results obtained from these methods (Figueroa and Buening, 1995; Zarlenga and Higgis, 2001). Real-time PCR is easy to perform, less prone to contamination and reduces the time and labour required for attainment of results (Bell and Ranford-Cartwright, 2002; Jaton et al., 2006). A quantitative real-time PCR (qPCR) assay based on the 18S rRNA gene developed by Sibeko et al. (2008) is currently used, together with other diagnostic tests, for the diagnosis of *T. parva* infections in cattle and buffalo in South Africa. However, the sensitivity of this test in diagnosing *T. parva* from buffalo that are co-infected with *Theileria* sp. (buffalo) is compromised as the 18S rRNA genes of both species are amplified by the *T. parva*-specific primer set (Sibeko et al., 2008; Chaisi et al., 2011; Mans et al., 2011; Pienaar et al., 2011). Alternative assays, based on more informative molecular markers, are needed to accurately detect and differentiate between pathogenic and non-pathogenic *Theileria* species in cattle and buffalo. Since PCR performance is proportional to the number of amplification targets present in a sample, targeting a repeated gene should increase the sensitivity of PCR assays (Criado et al., 2006).

A nested qPCR based on the cytochrome oxidase subunit (cox) III gene was developed for simultaneous detection and differentiation of six *Theileria* spp. in cattle samples by melting curve analysis (Janssens, 2009). This assay was further modified to include the detection of *Theileria* sp. (buffalo) from buffalo (results not published). Cox is the terminal component of the mitochondrial respiratory chain, it catalyzes the reduction of oxygen to water, producing ATP via oxidative phosphorylation and therefore allows the cell to utilise energy and oxygen (Cannino et al., 2004; Lienard et al., 2006). It is composed of 13 sub-units, and cox III is one of its three mitochondrial components (Schmidt et al., 1997).

The qPCR assays by Sibeko et al. (2008) and Janssens et al. (2009) are both based on fluorescence resonance energy transfer (FRET) technology. This involves the use of sequence-specific oligonucleotide (hybridization) probes that are labelled with fluorescence dyes (Reuter et al., 2005). Hybridization probes provide a simple way of analysing sequence variations using a single reaction and one set of probes as both amplification and hybridization occur in the same reaction (Caplin et al., 1999). When both probes have hybridized to the PCR product, and are heated by slowly raising the temperature, the donor (sensor) probe absorbs light and transfers the resonance energy to the acceptor (anchor). The temperature (T_m) at which the hybridization probes are melted off the DNA strand can then be quantitatively measured in real-time by melting curve analysis (Caplin et al., 1999; Reuter et al., 2005). The sensor probe covers the variable target sequence, and therefore determines the T_m, and the anchor probe produces the fluorescent signal. If the sensor probe is designed to perfectly fit the DNA strand, then it melts off at a higher temperature than when there is a mismatch (Reuter et al., 2005). Generally, a T_m difference of more than 1.5ºC from the standard curve is an indication of the presence of a mutation (Reuter et al., 2005), and a single mismatch between the sensor probe and mutant will reduce the T_m by about 5-8ºC (Landt and Nitsche, 1999). To improve the discriminative power of the sensor probe, the T_m of the sensor probe should be lower than that of the anchor probe, and this can be achieved by: (i) shortening the sequence of the sensor probe, (ii) increasing the G/C content of the anchor probe, or (iii) modifying the sensor probe with locked nucleic acids (LNAs) on the polymorphic position (Caplin et al., 1999; Op den Buijsch et al., 2005).

The aim of the current study was to evaluate the modified cox III qPCR assay for use in the detection and differentiation of *Theileria* spp. in mixed infections in buffalo. The blood samples analysed originated from buffalo from South Africa and Mozambique. The results obtained were compared to those obtained by the reverse line blot (RLB) hybridization assay (Gubbels et al., 1999) which is also used to simultaneously detect and differentiate *Theileria* spp. in mixed infections. The results of the cox III qPCR assay were also compared to those of the 18S rRNA gene qPCR (Sibeko et al., 2008) for the specific detection of *T. parva*.

7.3 Materials and methods

7.3.1 Samples and DNA extraction

A total of 224 samples (buffalo=220: cattle= 4), collected either in EDTA tubes or on filter paper, were analysed. The buffalo samples originated from African buffalo in different game parks in South Africa and Mozambique (Table 7.1). Four cattle samples were obtained from the Agricultural Research Council-Onderstepoort Veterinary Institute (ARC-OVI). DNA was extracted using the QIAamp® DNA Extraction Kit (QIAGEN, Southern Cross Biotechnologies), or the High Pure PCR Template Preparation kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturers' protocols, and stored at -20°C until further analysis.

Table 7.1: Origin and no. of samples analysed by the modified cox III qPCR assay

Place of origin	**Country (Province)**	**No. of samples**
Kruger National Park (KNP)	SA* (Mpumalanga and Limpopo)	54
Hluhluwe-iMfolozi Game Park (HIP)	SA (KwaZulu-Natal)	100
Agricultural Research Council - Onderstepoort VeterinaryInstitute (ARC-OVI)	SA (Gauteng)	4
Greater Limpopo Transfrontier Game Park (GLTP)	Mozambique	33
Others:	SA	
Addo Elephant Game Park (AEGP)	(Eastern Cape)	13
MarakeleNational Park	(Limpopo)	5
IthalaNational Park	(KwaZulu-Natal)	8
VaalbosNational Park	(Northern Cape)	6
Kwanare Game Park	(Mpumalanga)	1
TOTAL		**224**

* SA – South Africa

7.3.2 Polymerase Chain Reaction

A nested PCR protocol was used for the amplification of a fragment of the cox III gene of the parasite. Forward primer F3Cox (5'-AAG ATG AAT CCG ATT TGA TGA-3') and reverse primer MJCox (5'-AAA TGG ACT ATG TAA GTT AAC CTAT-3') were used in a primary conventional hot start PCR reaction. The reaction mixture contained 1 µl yellow sub (GENEO BioProductions, Hamburg, Germany), 5 µl of 1X Go Taq buffer (Promega), 1.65 mM $MgCl_2$, 2.5 mM dNTPs, 0.4 µM of each primer, 0.5 U *Taq* Polymerase (Promega), 5 µl (~ 100 ng) DNA and PCR grade water to a total volume of 25 µl. The cycling conditions included an initial hold at 84^{0}C for 10 s, initial denaturation at 92^{0}C for 4 min, amplification of 25 cycles each of denaturation at 92^{0}C for 30 s, annealing at 56^{0}C for 45 s and extension at 72^{0}C for 60 s, and a final extension at 72^{0}C for 10 min. All primary PCR products were analysed by a nested cox III qPCR assay protocol using the Rotor Gene 3000 (Corbett Research, Australia). Each reaction contained 0.5 µl of the primary PCR product, 1X Go Taq buffer (Promega), 1.65 mM $MgCl_2$, 2.5 mM dNTPs, 0.66 µM of primer Fcox(5'-CAA CAT TGT TAA AGC TAT CCA A-3') and 0.13 µM of primer nRCox(5'-TTA TAG TAC AGG ATT AGA TAC-3'), together with 0.5 µM each of the modified Anchor probe Cox1-6FAM (5'-ATT GGa tga cat taT AtT tct ata ttt taa CaG GAc-3') and Sensor probe Cox1-Cy5 (5'-Att caT tac acG Tat gtg Ctg gaa g-3'), 5U *Taq* polymerase and water to a total volume of 25 µl. Capital letters in the anchor and sensor probe sequences represent locked nucleic acids (LNAs). The programme included a hold at 95^{0}C for 15 min, 40 cycles each of denaturation at 95^{0}C for 30 s, annealing at 56^{0}C for 45 s, extension at 72^{0}C for 60 s. Melting curves were generated by heating the samples from 33^{0}C to 99^{0}C with a heating rate of 1^{0}C/min. Fluorescence was measured at 640 nm.

Plasmid DNA from cox III clones (obtained from the Department of Animal Health, Institute of Tropical Medicine, Antwerp, Belgium) of the following species were used as positive controls: *Theileria* sp. (buffalo) clone 1.5 originated from a buffalo isolate from South Africa; *T. parva* Katete clone 1.5 was obtained from a bovine isolate (used as a vaccine strain) from the Eastern province of Zambia; *T. taurotragi* N355 clone 2.7 was obtained from a bovine isolate from the Eastern province of Zambia; *T. buffeli* M2138 clone 538 was from an imported bovine in Butare, Rwanda; *T. velifera* C914 clone 2.8 and *T. mutans* C914 clone 2.2 were from a mixed infection sample obtained from a bovine in the Eastern province of Zambia. Molecular grade water was used as a negative control.

7.3.3 Cloning and sequencing

Based on the qPCR results, 17 samples were selected for further characterization. Primary PCR products were purified using the High Pure PCR product purification kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. The samples originating from the AEGP (n = 7) had single *T. buffeli* infections and their cox III genes were amplified and PCR products were directly sequenced. The PCR product from the sample from buffalo KNP102 (Sibeko et al., 2008) was directly sequenced and also cloned before sequencing, and PCR products from the remaining nine samples were cloned prior to sequencing. Ligations and transformations were done using the pCR2.1TOPO cloning vector (Invitrogen, Carlsbad, USA) as recommended by the manufacturer. Ninety-three recombinants obtained from these samples were screened using the cox III qPCR assay as described above. Based on the qPCR results, desired clones were then cultured overnight in LB-broth, after which they were re-grown on fresh agar plates and sent for sequencing. Plasmid extraction and sequencing were done at the Genetic Service Facility, University of Antwerp, Belgium and at Inqaba Biotechnologies, South Africa. In South Africa, sequencing reactions were done using the ABI Big Dye Terminator Cycle Sequencing kit version 3.1 (Applied Biosystems, Foster City, CA) using ~350 ng of plasmid DNA and 2 pmol each of primers F3 Cox, MJCox3 and nRcox.

7.3.4 Sequence and phylogenetic analysis

The cox III sequences were assembled and edited using GAP4 of the Staden software package (version 1.6.0 for Windows) (Staden et al., 2000). MAFFT version 5 (Katoh et al., 2005) was used to align the new sequences with cox III gene sequences of the control clones, and with published *Theileria* cox III gene sequences from GenBank (*T. parva* Z23263, *T. parva* AB499089, *T. orientalis* AB499090, *T. annulata* U32225). The alignment was manually edited using BioEdit (version 7) (Hall, 1999). A BLASTn homology search of GenBank was done using the consensus sequences. The genetic distances between the sequences were estimated by determining the number of base differences between sequences using MEGA4 (Tamura et al., 2007). Phylogenetic trees were constructed using MEGA4 for neighbor-joining analysis with 1000 bootstrap replicates (Felsenstein, 1985); PAUP* (v4b10) (Swofford, 2003) for maximum-parsmony and maximum likelihood methods, and MrBayes v.3.1.2 (Ronquist and Huelsenbeck, 2003) for Bayesian inference, accessed via the Computational Biology Service Unit, Cornell University (http://mafft.cbsuapps.tc.cornell.edu/mrbayes.aspx). The TrN + I + G substitution method was determined as the best fit model by Modeltest v.3.7 (Posada et al., 1998) and used in the likelihood

and Bayesian analyses.The trees were rooted using the cox III gene sequence of *Theileria annulata* (U32225) and consensus trees were edited using MEGA 4.

7.3.5 Comparison of the cox III qPCR assay with the RLB assay and the 18S rRNA qPCR

The samples (n = 224) were analysed by the RLB hybridization assay as described by Gubbels et al. (1999), for the simultaneous detection and differentiation of *Theileria* spp. The *Theileria* and *Babesia* species- and genus-specific probes used were as previously described (Chapter 3; Chaisi et al., 2011). Additionally, 206 samples were analysed for the specific detection of *T. parva* using the 18S rRNA qPCR assay, as described by Sibeko et al. (2008). The occurrence of *T. parva* in these samples was compared to that of the coxIII pPCR and RLB hybridization assays. For the 18S rRNA qPCR assay, the amplification mixture consisted of 4 µl of 10X LightCycler-FastStart DNA Master^{Plus} Hybridization Probes mix (Roche Diagnostics, Mannheim, Germany), 0.5 µM of each of *T. parva* specific forward primer (5'-CTG CAT CGC TGT GTC CCT T-3') and *Theileria* genus-specific reverse primer (5'-ACC AAC AAA ATA GAA CCA AAG TC-3'), 0.1 µM of each hybridization probe (*T. parva* anchor (5'-GGG TCT CTG CAT GTG GCT TAT-FL), *T. parva* sensor (5'-LCRed640-TCG GAC GGA GTT CGC T-PH), *Theileria* genus anchor (5'-AGA AAA TTA GAG TGC TCA AAG CAG GCT TT-FL) and *Theileria* genus sensor (5'-LCRed705-GCC TTG AAT AGT TTA GCA GCA TGG AAT-PH), 1 U uracil deoxy-glycosylase (UDG) and 2.5 µl (~ 37.5 ng) of template DNA in a final volume of 20 µl (Sibeko et al., 2008). Cycling was done using a LightCycler® v2 (Roche Diagnostics, Mannheim, Germany). The cycles include UDG activation at 40°C for 10 min, *Taq* DNA polymerase activation at 95°C for 10 min, 45 cycles of three steps each of denaturation at 95°C for 10 s, annealing at 58°C for 10 s, and extension at 72°C for 15 s. Melting curve analysis was performed by heating the samples from 40°C to 95°C with a heating rate of 0.2°C/s, and fluorescence values were measured at 640 and 705 nm.

7.4 Results

7.4.1 cox III qPCR results

Amplicons of approximately 1000 bp were obtained from primary PCR amplification of the cox III gene of *Theileria* spp. The hybridization probes used in the cox III real-time PCR assay allow for the detection and discrimination of the different species based on differences in their melting temperatures (T_m). The sequences of the modified anchor and sensor probes and *Theileria* spp. controls are indicated in Figure 7.1(a). Melting peak analysis was used to identify the different species, based on the comparison of their melting peaks with those of the control clones as illustrated in Figure 7.1(b). As the melting peaks can shift slightly from run to run, the mean and standard deviation of the T_m of the control plasmids and analysed samples were determined.

Theileria parva and *Theileria* sp. (buffalo) were the most commonly detected species in the field samples from buffalo and cattle, with prevalences of 83.5% and 55.8%, respectively (Figure 7.2). *Theileria taurotragi*, *T. buffeli* and *T. mutans* were identified in 1.8%, 5.8% and 2.2% of samples, respectively (Figure 7.2). *Theileria velifera* was not identified in any of the samples, and 4.5% of the samples were negative or below the detection limit of the assay. Additionally, 17% of the samples had non-specific peaks which were located between those of *Theileria* sp. (buffalo) and *T. velifera* or *T. taurotragi* and *T. mutans* (Figure 7.1c).

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                        MOD-anchor probe                    MOD-sensor probe
                        ATTGGatgacattaTAtTtctatattttaaCaGGAc  AttcaTtacacGTatgtgCtggaag
ZAM/C914/2.2    acaattattggatgacattgtatttctatattttaacaggacttcattcattacacgtatgtgttggaagtgtatttgtacttat  T. mutans
ZAM/N355/2.7    .t.....................a.....t.t.....................g..t.......c....g..a.c.....gtag..  T. taurotragi
SA/buffalo/1.5  .t.....................a..c..t.t.....................c.gt.t...........g..a.t......t.a..  Theileria sp.buffalo)
ZAM/Kat/1.1     .t.....................a..c..t.t.....................g.....t.......cc...g.ca.t........a..  T. parva
ZAM/C914/2.8    .t..c.....................c.a..c..t.....t.....c.t.........a.a.........c......t.a..  T. velifera
RWA/M2138/538   .tg.....a.....a.....t.....t...t.a....t.....t.....a...t.....tctt.a..  T. buffeli
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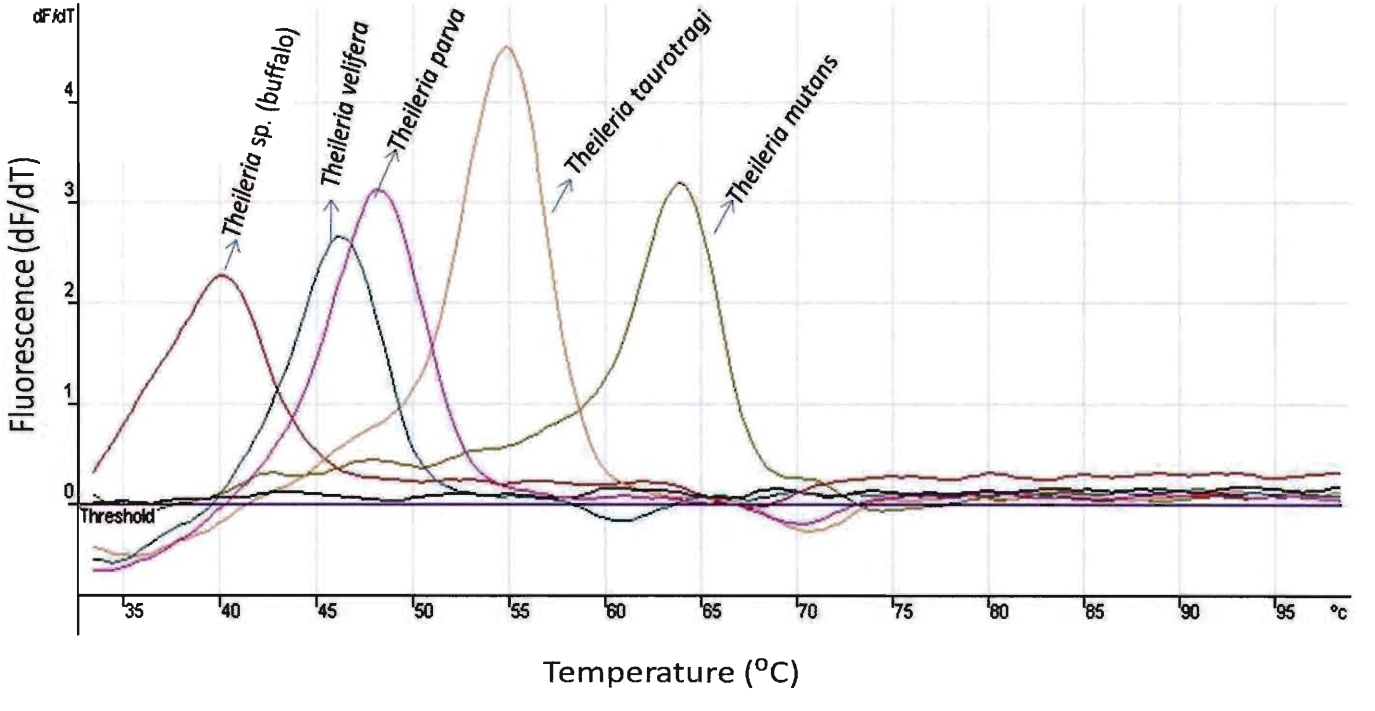
(a)



(b)

(c)

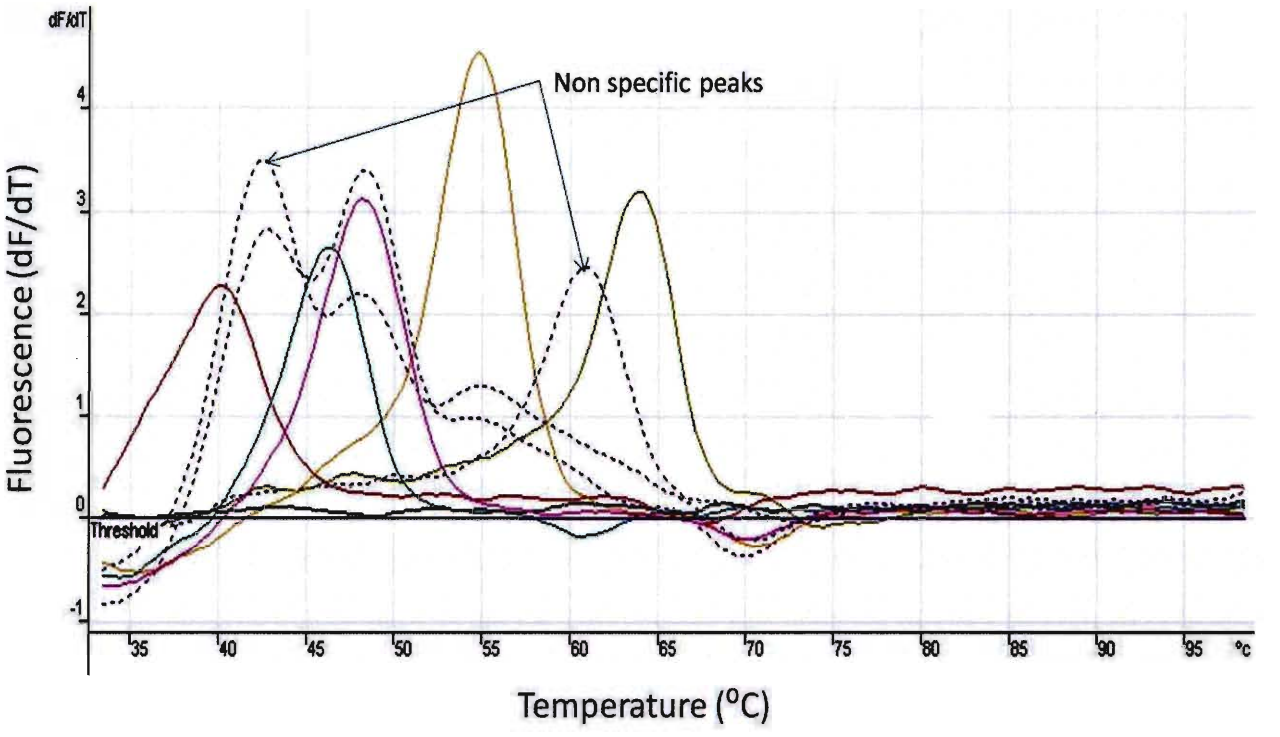


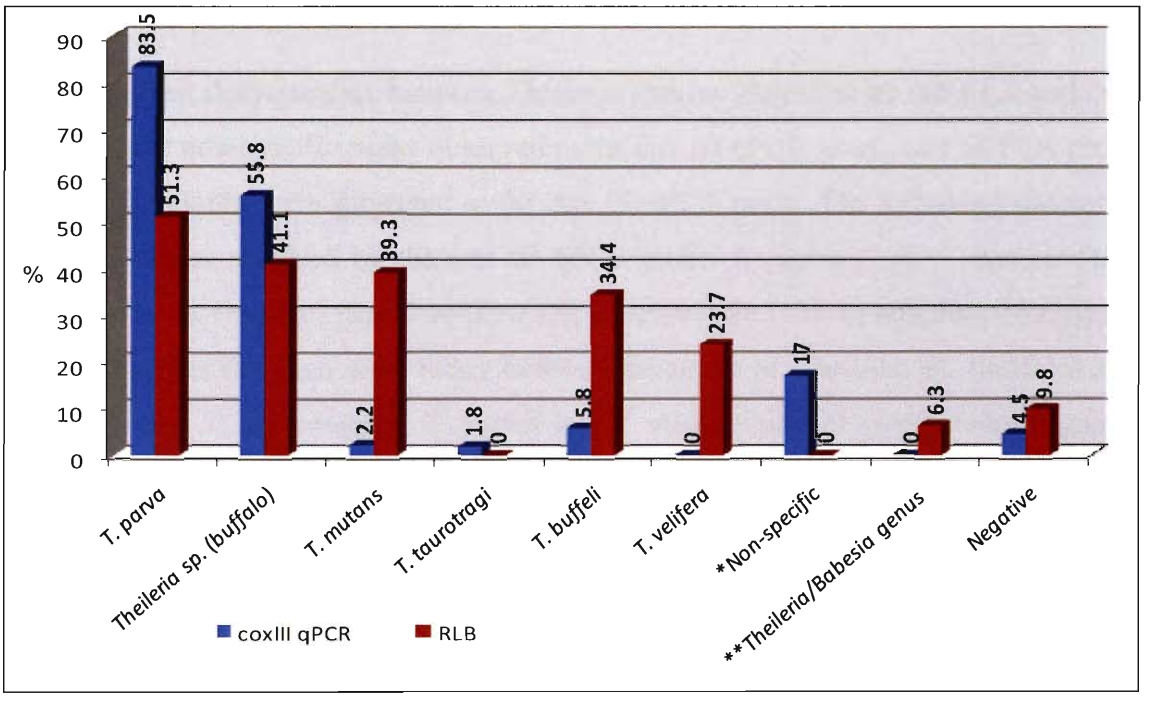
Figure 7.1: (a) Sequence alignment showing the number of mismatches in the modified FRET anchor and sensor probe areas in the different species. (b) Melting curve analysis of the cox III gene plasmid controls of *Theileria* spp. as determined by the cox III qPCR assay. Melting peaks shown are for *Theileria* sp. (buffalo) (39.7 ± 0.5^{0}C), *T. velifera* (46.0 ± 0.4^{0}C), *T. parva* (48.4 ± 0.3^{0}C), *T. taurotragi* (54.7 ± 0.8^{0}C), and *T. mutans* (63.9 ± 0.4^{0}C). The melting peak of *T. buffeli* (53.7 ± 0.1^{0}C) is not shown. (c) Non-specific peaks (arrows) were observed from some samples. No flourescence was detected from the water control.

7.4.2 Comparison of the cox III qPCR assay with the 18S qPCR and RLB hybridization assays

The cox III qPCR results were compared to those obtained by the RLB hybridization assay for the simultaneous detection and differentiation of *Theileria* spp. in buffalo and cattle (n = 224). *Theileria parva* and *Theileria* sp. (buffalo) were also identified as the most commonly occurring species by the RLB assay (Figure 7.2). However, the RLB assay detected more infections of *T. mutans* and *T. buffeli* than the cox III qPCR assay, 23.7% of samples were positive for *T. velifera* and no *T. taurotragi* infections were detected by the RLB assay. Of the 14 samples that only had a *Theileria/Babesia* genus-specific signal on the RLB assay, 11 showed mixed *Theileria* spp. infections and 3 were negative on the cox III qPCR assay (Figure 7.2a).

The cox III qPCR assay results were also compared with those of the RLB and 18S qPCR assays (n = 206) for the specific detection of *T. parva*. This species was detected in 107 (51.9%), 157 (79.2%) and 178 (86.4%) of the 206 samples analysed by the RLB, 18S qPCR and cox III qPCR assays, respectively (Figure 7.2b).

(a)



(b)

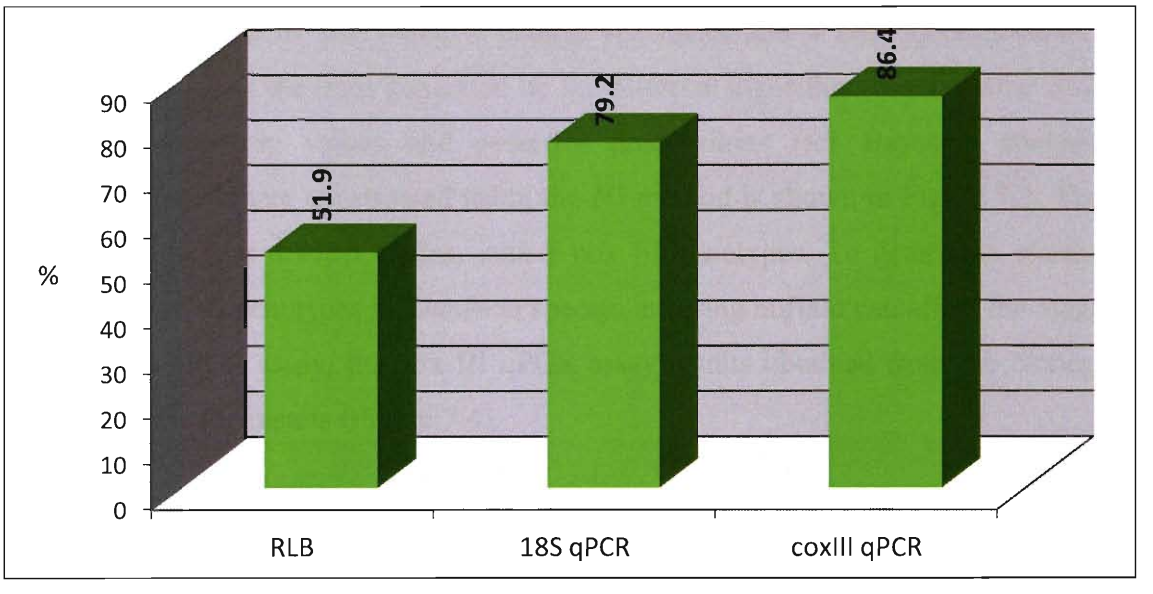


Figure 7.2: (a) The occurrence of *Theileria* species infections in buffalo and cattle samples from South Africa and Mozambique as determined by the RLB and cox III qPCR assays (n = 224). The number of samples with non-specific melting temperatures on the cox III qPCR assay (*) and those that hybridized only with the *Theileria/Babesia* genus-specific probes using the RLB assay (**) are shown, (b) Comparison of the RLB, 18S qPCR and cox III qPCR assays for detection of *T. parva* (n = 206).

7.4.3 Sequencing and phylogenetic results

Because of the marked discrepancies between *Theileria* species identified by the RLB and cox III qPCR assays, and the non-specific peaks observed in the cox III qPCR assay, cox III PCR products were cloned and the clones were subjected to the cox III qPCR assay. The following species were detected from 93 clones analysed by the cox III qPCR assay: *T. parva* (13); *T. mutans* (10); *T. buffeli* (7); *Theileria* sp. (buffalo) (9); *T. velifera* (2); *T. taurotragi* (17). In addition, 14 clones had non-specific melting peaks which were either between the peaks of *Theileria* sp. (buffalo) and *T. velifera*, *T. buffeli* and *T. taurotragi* or *T. buffeli* and *T. mutans,* and 21 clones were negative or below detection limit. Based on these results, selected clones were sequenced.

A total of 26 new cox III gene sequences were obtained from 17 samples that were selected for sequencing. A BLASTn homology search revealed the closest homology with cox III gene sequences of *T. parva* (Z23263) (81 -100%), *T. parva* (AB499089) (79 - 100%) and *T. orientalis* (AB499090) (78 - 88%). Phylogenetic trees were constructed from a total of 36 cox III sequences (26 new sequences obtained in this study, 6 control sequences and 4 sequences obtained from GenBank). The groupings in the trees generated by the different algorithms were similar and were supported by high bootstrap values and posterior probabilities (for Bayesian analysis). A representative phylogenetic tree constructed using the NJ method is shown in Figure 7.3. The new sequences grouped into four distinct clades, with 9 cox III genotypes. To determine whether the presence of diverse cox III genotypes in *Theileria* species infecting buffalo can affect the diagnostic results of the cox III qPCR assay, the cox III qPCR assay results obtained from the clones were compared to the sequencing results (Figure 7.4).

The *T. mutans* group (clade A) contained four genotypes and was the most polymorphic group. Sequence KNP/C8/9.1 from a buffalo sample, was identical to the *T. mutans* control cox III sequence ZAM/914/2.2 from a bovine sample. Clone KNP/C8/9.1 tested positive for *T. mutans* by the cox III qPCR assay. The original sample, KNP/C8, tested positive for *T. mutans* and *T. velifera* when analysed by the RLB hybridization assay (Figure 7.4), but no *T. velifera* cox III clones were obtained from this sample. The three *T. mutans*-like cox III genotypes were designated *T. mutans*-like A – C (Figure 7.3). All of the clones from which these sequences were obtained had non-specific melting peaks that were between those of *T. taurotragi* and *T. mutans* when analysed by the cox III qPCR assay (Figure 7.4).

The sequencing results correlated with the results of the RLB assay as these samples tested positive for *T. mutans*, although the signal in sample HIP/A2 was very weak. The four *T. mutans*-like A sequences were all derived from buffalo samples. The two *T. mutans*-like B sequences from clones KNP/K4/3.8 and OVI/8227/4.10, were derived from a buffalo sample and a bovine sample respectively, and the *T. mutans*-like C sequences (4) were buffalo-derived. Nucleotide differences of 1-3 bp in the modified sensor probe between *T. mutans* and *T. mutans*-like cox III genotypes resulted in a shift in T_m of up to 8°C (Figure 7.4), and as a result, clones with *T. mutans*-like cox III sequences yielded non-specific melting peaks when analysed by the cox III qPCR assay.

Clade B consisted of cox III gene sequences of the pathogenic *Theileria* spp. (*T. taurotragi* and *T. parva*) as well as those of *Theileria* sp. (buffalo). None of the new sequences grouped with the *T. taurotragi* cox III control sequence (ZAM/N355/2.7) obtained from a bovine sample from Zambia. Although sequence OVI/8227/4.7, obtained from a bovine at the OVI, was most closely related to the *T. taurotragi* sequence, its identity could not be established and it was therefore indicated as inclusive. This clone had a melting peak between *Theileria* sp. (buffalo) and *T. velifera* when tested using the cox III qPCR assay, and the field sample was positive for *T. mutans* by the RLB assay. The sequence from clone HIP/A2/1.9 was identical to the cox III sequence of the *Theileria* sp. (buffalo) control clone (SA/buffalo/1.5). The *T. parva* cox III sequences (KNP/102 and KNP/102/6.2) from buffalo 102 were identical to the *T. parva* (Muguga stock) cox III sequences from cattle, Z23263 (Kairo et al., 1994) and AB499089 (Hikosaka et al., 2010), and to the cox III sequence (ZAM/Kat/1.1) of the *T. parva* control used in this study (Figure 7.3). All three clones tested positive for *T. parva* by the cox III PCR and *T. parva* was identified in the original field samples using the RLB assay.

Clade C contained the *T. velifera* cox III sequences from cattle (ZAM/C914/2.8) and buffalo (HIP/A2/1.1), and *T. velifera*-like cox III sequences (KNP/K8/2.2, KNP/E2/8.5, HIP/A2/1.7) which were all derived from buffalo. In contrast, *T. velifera* was only identified (using the RLB assay) in two of the three field samples from which these clones were obtained (Figure 7.4). The clones of the three *T. velifera*-like cox III sequences had peaks between *T. taurotragi* and *T. mutans* when analysed by the cox III qPCR assay.

The last clade (D) was the *T. buffeli* group. The cox III sequences of the seven samples from the AEGP were identical and differed from the published cox III gene sequence of *T. orientalis* (AB499090) (Hikosaka et al., 2010) and the sequence of the *T. buffeli* control clone (RWA/M2138/538). These sequences were therefore designated as *T. buffeli*-like cox III sequences. Although there are three bp differences in the sensor probe areas of RWA/M2138/538 and the AEGP sequences, their melting temperature was almost identical (0.3°C difference). All the field samples from which these sequences were derived tested positive for *T. buffeli* using the RLB assay.

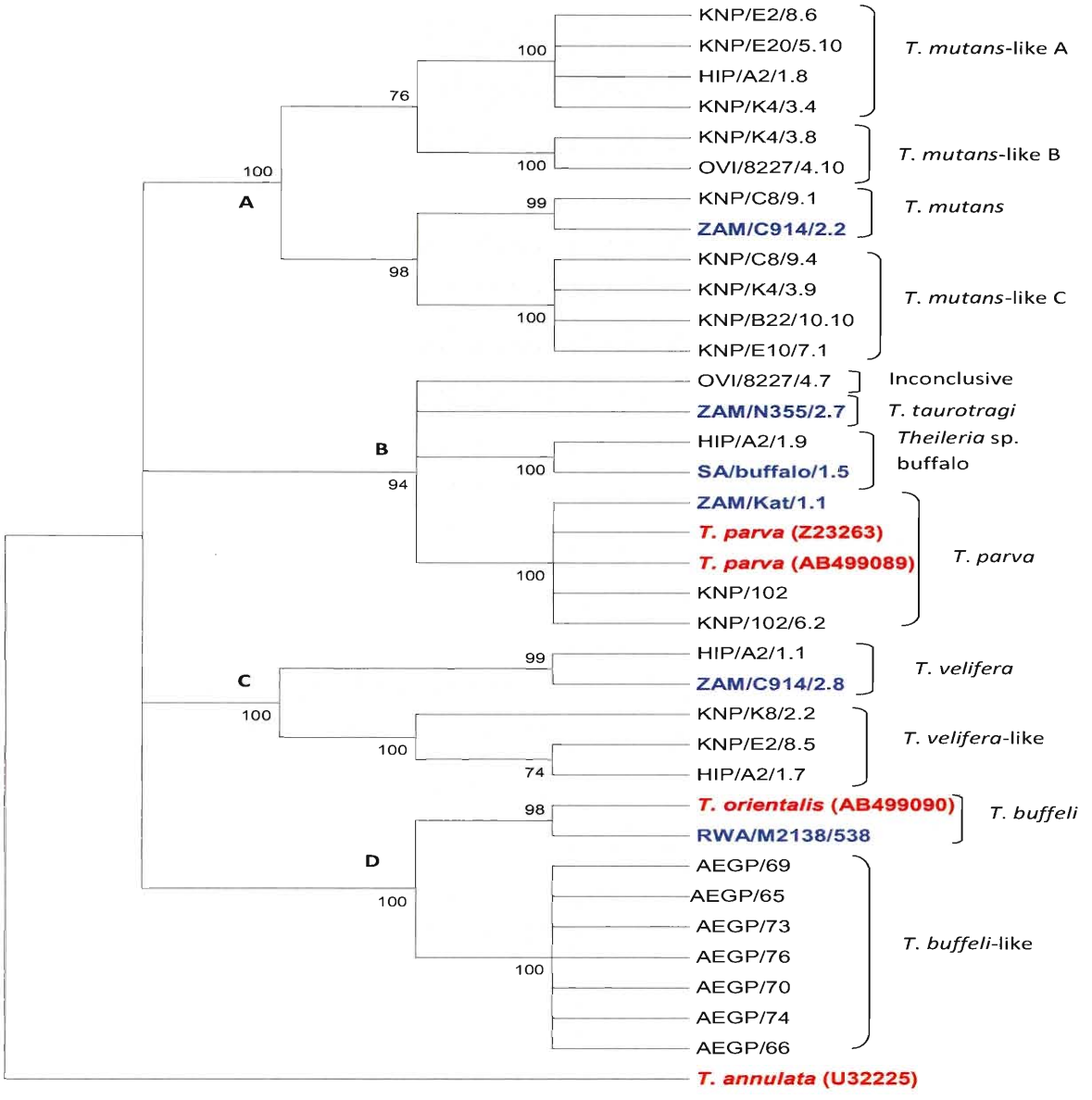
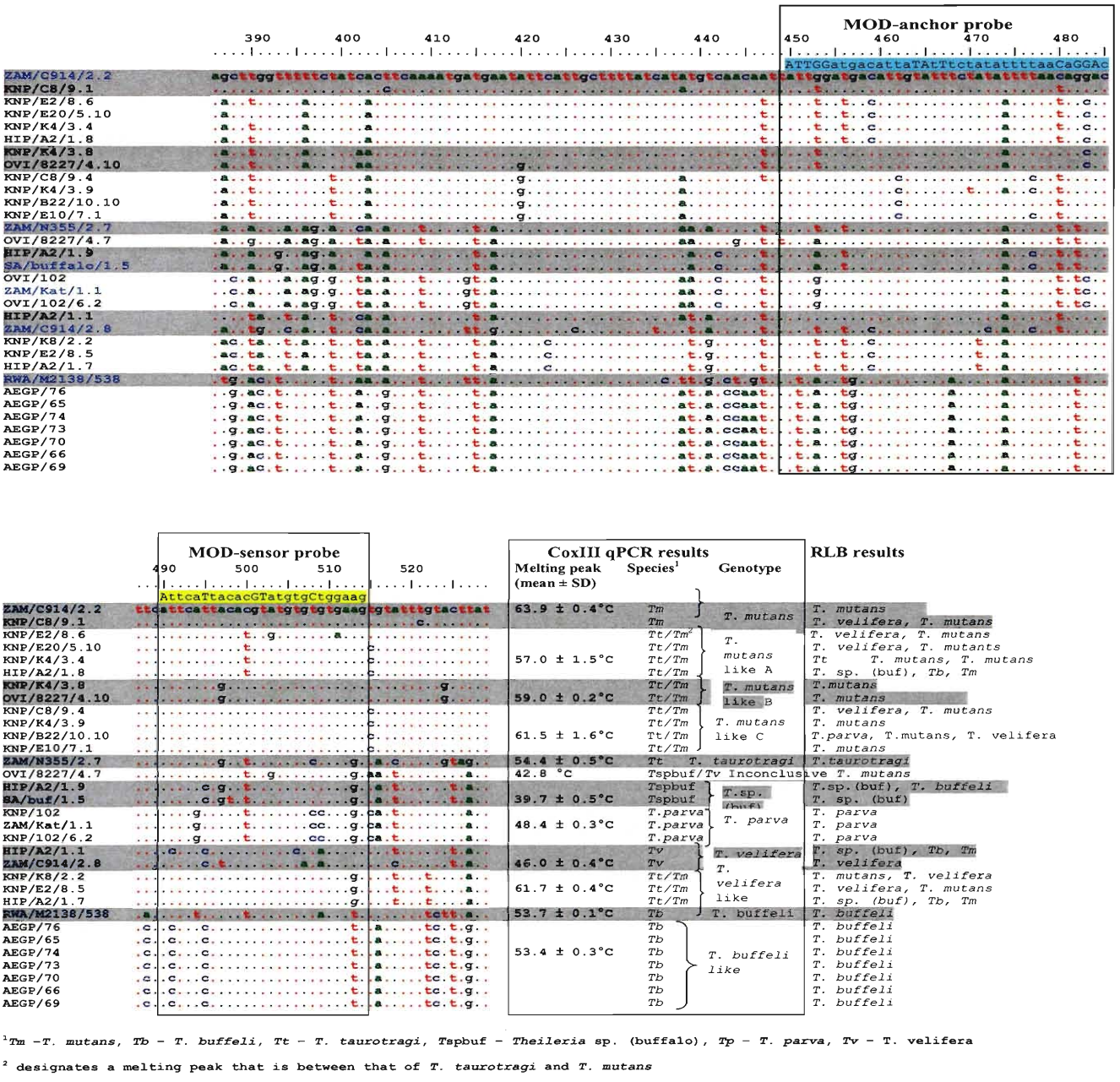


Figure 7.3: Phylogenetic relationships of the cox III gene sequence variants of *Theileria* spp. of buffalo and cattle in South Africa identified in this study (black) with *Theileria* control sequences (blue) and published *Theileria* spp. (red) as indicated by neighbor joining analysis. Bootstrap values indicate the degree of support for each cluster. The tree was outgroup rooted using the cox III gene sequence of *T. annulata.*



[1]*Tm* – *T. mutans*, *Tb* – *T. buffeli*, *Tt* – *T. taurotragi*, *Tspbuf* – *Theileria* sp. (buffalo), *Tp* – *T. parva*, *Tv* – T. velifera

[2] designates a melting peak that is between that of *T. taurotragi* and *T. mutans*

Figure 7.4: Sequence alignment of the cox III gene of *Theileria* spp. indicating the sequences of the modified anchor (light blue) and sensor (yellow) probes. Cox III sequences were obtained from clones from control samples and clones from selected African buffalo samples. Differences are based on the cox III sequence of *T. mutans* (ZAM/C9142.2). The cox III qPCR result obtained for each clone is shown along with the cox III genotype identified by phylogenetic analysis. The RLB results obtained from the sample from which the cox III clones were derived are also shown.

7.5 Discussion and Conclusion

FRET technology has previously been used for the development of diagnostic assays to simultaneously identify co-infecting piroplasmids (*Theileria* and *Babesia* spp.) in different hosts. Criado-Fornelio et al. (2009) developed a qPCR assay, based on the 18S rRNA gene, for the simultaneous identification of *Babesia bovis*, *Babesia divergens*, *Babesis major*, *Babesia bigemina*, *Theileria annae* and an unidentified *Theileria* sp. in bovines. The qPCR by Wang et al. (2010), also based on the 18S rRNA gene, differentiates *B*abesia *gibsoni*, *Babesia canis canis*, *Babesia canis vogeli* and *Babesia canis rossi* in canines. In addition to the identification of *T. parva* in infected animals, the 18S qPCR by Sibeko et al. (2008) can simultaneously detect *T. taurotragi* and *T. annulata* when the *Theileria*-genus primer set is used. The cox III qPCR assay of Janssens (2009) was developed for the simultaneous detection and differentiation of *T. parva* and five co-infecting *Theileria* spp., namely *T. annulata*, *T. velifera*, *T. taurotragi*, *T. mutans* and *T. buffeli* in infected cattle based on differences in their melting temperatures (Janssens, 2009). This assay was later modified, by the development of new primer and probe sets, to include the detection of *Theileria* sp. (buffalo) in buffalo (unpublished results). In this study the modified cox III qPCR assay was compared with the 18S qPCR assay for the specific detection of *T. parva*, and with the RLB assay for the 'universal' detection and discrimination of *Theileria* spp. in cattle and buffalo samples.

The analytical sensitivities of the qPCR assays for the detection of *T. parva* were determined as 100% at parasitemia of 8.79×10^{-4}% for the 18S qPCR assay (Sibeko et al., 2008), and between 4.1×10^{-5} and 4.1×10^{-4}% for the cox III qPCR assay (Janssens, 2009), indicating that the latter assay might be slightly more sensitive than the former. More *T. parva* infections were identified by the cox III qPCR than the 18S rRNA assay. Both qPCR assays have previously been reported as being more sensitive than the 18S- and cox III RLFP-PCR assays in detecting *T. parva* (Sibeko et al., 2008; Janssens, 2009).

The cox III qPCR assay was also more sensitive than the RLB assay in the detection of *T. parva* and *Theileria* sp. (buffalo) infections. Real-time PCR has previously been reported to be more sensitive than the RLB assay in detecting *Theileria* and *Babesia* spp. (Sibeko et al., 2008; Bhoora et al., 2009). Additionally, the cox III qPCR is less laborious, less time-consuming and more cost-effective than the RLB assay as it requires the use of a single hybridization probe pair to detect all the *Theileria* spp., whereas the RLB requires a probe for each species or genotype that is identified.

The cox III qPCR assay has the potential of replacing or complementing the RLB assay for the simultaneous detection of *Theileria* spp. of cattle and buffalo in the future. However, the cox III qPCR assay used in this study was shown to be less specific than the RLB in the detection of other *Theileria* spp. that infect the African buffalo.

Sequence and phylogenetic analyses of the cox III gene in our study indicated the presence of a single *T. parva* genotype in cattle and buffalo, and therefore the cox III qPCR assay can specifically detect *T. parva* infections in these hosts where the parasitemia is above the detection limit of the assay. However, sequence polymorphism in the cox III genes of the other *Theileria* species decreases the specificity of the assay for these species; hence their apparent low prevalences as indicated by the cox III qPCR results, and the discrepancies between cox III qPCR and RLB assay results.

The identification of *T. taurotragi* by the cox III qPCR assay in buffalo was unexpected. To our knowledge this species has never been isolated from the African buffalo. In addition, the RLB assay did not detect *T. taurotragi* in any of these samples. The RLB result was confirmed by sequence analysis: none of the cox III sequences derived from buffalo samples grouped together with the *T. taurotragi* control sequence. Sequencing and phylogenetic analyses of clones that had non-specific cox III melting peaks between the *T. taurotragi* and *T. mutans* peaks were identified as *T. mutans*-like genotypes. This limitation of the cox III assay may be overcome by designing probes from a region within the cox III gene that is more variable between the two species. Although the cox III qPCR assay can distinguish between the different *Theileria* spp., failure of the assay to accurately distinguish between the different species variants is another limitation of the test.

As with the 18S rRNA gene (Chapters 4, 6), the greatest heterogeneity in the cox III gene was observed within the *T. mutans* group. Although a direct comparison between the two genes could not be made as different samples (with the exception of sample KNP/B22) were analysed and pure parasite stocks are not available, we also identified a *T. mutans* cox III genotype (identical to cox III *T. mutans* from cattle) and three *T. mutans*-like cox III genotypes. However, unlike the 18S rRNA gene where all the genotypes were exclusively obtained from buffalo samples, we identified a *T. mutans*-like B cox III genotype from a cattle sample (OVI/8227/4.10).

Further studies are needed to determine if sequence OVI/8227/4.7 represents a unique sequence in cattle as the clone had a unique melting peak that was between those of *Theileria* sp. (buffalo) and *T. velifera*. Although we identified a single *T. parva* genotype in both cattle (Muguga strain) and

buffalo, analysis of more clones might reveal the presence of more *T. parva* cox III genotypes as Mans et al. (2011) identified 8 cattle and 3 buffalo *T. parva* 18S rRNA genotypes.

The identification of a *T. velifera* genotype in cattle and buffalo, and a *T. velifera*-like genotype in buffalo is concurrent with our previous results on the 18S rRNA gene (Chapter 4). Low parasitemia might explain why the cox III assay failed to identify *T. velifera* from sample HIP/A2 by RLB and cox III qPCR assays. Similarly, the very faint *T. velifera* signal obtained from sample KNP/K8 might be due to low parasitemia. Sequence variations between the *T. velifera* control sequence and the *T. velifera*-like sequences explains the failure of the cox III assay to identify *T. velifera* in the field samples. All three *T. velifera*-like sequences had melting peaks between those of *T. buffeli* and *T. mutans* by the cox III qPCR. This result was obtained with the *T. mutans*-like C sequences and might be due to the fact that there is only one nucleotide difference in the sensor probe area of two genotypes.

The design of species-specific primers and probes has been restricted by the lack of cox III sequence data. When this study commenced, the only *Theileria* spp. for which cox III sequences were available in GenBank were *T. parva*, *T. annulata*, *T. equi* and *T. orientalis*. Sequence data obtained from this study will therefore allow for the design of new primers and probes for effective differentiation between the different species and their variants.

In conclusion, there is extensive sequence variation within the cox III gene of *Theileria* spp. of the African buffalo. Although the gene is a good marker in phylogenetic studies of closely related species, it might not be a suitable gene for use in a diagnostic assay, particularly in *Theileria* spp. of buffalo where there is a lot of variation. The observed microheterogeneity within genotypes is possibly due to *Taq* polymerase error (Zahler et al., 1998; Aktas et al., 2007). It is possible that analysis of more samples could reveal more variation as the cox III gene is a fast evolving gene. Whether the observed variation represents new species or just variants of known species is uncertain and cannot be determined based on the results of a single gene. Our results further indicate the importance of the identification and characterization of all local genotypes of a gene before the development of diagnostic assays as suggested by Bhoora et al. (2009). The sensitivity and specificity of the cox III qPCR assay for the identification of benign and non-pathogenic *Theileria* spp. in the African buffalo could be improved by the development of primers from a conserved area of the gene and probes from variable areas of the gene.

7.6 References

AKTAS, M., BENDELE, K. G., ALTAY, K., DUMANLI, N., TSUJI, M., HOLMAN, P. J., 2007. Sequence polymorphism in the ribosomal DNA internal transcribed spacers differs among *Theileria* species. Veterinary Parasitology 147, 221-230.

BELL, A., RANFORD-CARTWRIGHT, L., 2002. Real-time quantitative PCR in parasitology. Trends in Parasitology 18, 338.

BHOORA, R., FRANSSEN, L., OOSTHUIZEN, M.C., GUTHRIE, A.J., ZWEYGARTH, E., PENZHORN, B.L., JONGEJAN, F., COLLINS, N.E., 2009. Sequence heterogeneity in the 18S rRNA gene within *Theileria equi* and *Babesia caballi* from horses in South Africa. Veterinary Parasitology 159, 112-120.

CANNINO, G., DI LIEGRO, C. M., DI LIEGRO, I., RINALDI, A. M., 2004. Analysis of cytochrome C oxidase subunits III and IV expression in developing rat brain. Neuroscience 128, 91-98.

CAPLIN, B.E., RASMUSSEN, R.P., BERNARD, P.S., WITTWER, C.T., 1999. The most direct way to monitor PCR amplification for quantification and mutation detection. Biochemica 1, 5-8.

CHAISI, M.E., SIBEKO, K.P., COLLINS, N.E., POTGIETER, F.T., OOSTHUIZEN, M.C., 2011. Identification of *Theileria parva* and *Theileria* sp. (buffalo) 18S rRNA gene sequence variants in the African Buffalo (*Syncerus caffer*) in southern Africa. Veterinary Parasitology 182, 150-162.

COLLINS, N. E., ALLSOPP, M. T. E. P., ALLSOPP, B. A., 2002. Molecular diagnosis of theileriosis and heartwater in bovines in Africa. Transactions of the Royal Society of Tropical Medicine and Hygiene 96, S217-S224.

CRIADO, A., MARINEZ, J., BULING, A., BARBA, J.C., MERINO, S., JEFFERIES, R., IRWIN, P.J., 2006. New data on epizootiology and genetics of piroplasms based on sequences of small ribosomal subunit and cytochrome b genes. Veterinary Parasitology 142, 238-247.

CRIADO-FORNELIO, A., BULING, A., PINGRET, J.L., ETIEVANT, M., BOUCRAUT-BARALON, C., ALONGI, A., AGNONE, A., TORINA, A., 2009. Hemoprotozoa of domestic animals in France: prevalence and molecular characterization. Veterinary Parasitology 159, 73-76.

FELSENSTEIN, J., 1985. Confidence limits on phylogenies: An approach using the bootstrap. Evolution 39, 783-791.

FIGUEROA, J. V., BUENING, G. M., 1995. Nucleic acid probes as a diagnostic method for tick-borne hemoparasites of veterinary importance. Veterinary Parasitology 57, 75-92.

GUBBELS, J.M., VOS, A.P., WEIDE, M., VISERAS, J., SCHOULS, L.M., VRIES, E., JONGEJAN, F., 1999. Simultaneous detection of bovine *Theileria* and *Babesia* species by reverse line blot hybridization. Journal of Clinical Microbiology 37, 1782-1789.

HALL, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acid Symposium Series 41, 95-98.

HIKOSAKA, K., WATANABE, Y., TSUJI, N., KITA, K., KISHINE, H., ARISUE, N.,PALACPAC, N.M., KAWAZU, S., SAWAI, H., HORII, T., IGARASHI, I., IANABE, K., 2010. Divergence of the mitochondrial genome structure in the apicomplexan parasites, *Babesia* and *Theileria*. Molecular Biology and Evolution 27, 1107-1116.

JANSSENS, M.E., 2009. Molecular biological tools for the immunization and diagnosis of *Theileria parva*. PhD. thesis. Faculteit Wetenschappen. Universiteit Anwerpen. Belgium.

JATON, K., BILLE, J., GREUB, G., 2006. A novel real-time PCR to detect chlamydia trachomatis in first-void urine or genital swabs. Journal of Medical Microbiology 55, 1667-1674.

KAIRO, A., FAIRLAMB, A. H., GOBRIGHT, E., NENE, V., 1994. A 7.1 kb linear DNA molecule of *Theileria parva* has scrambled rDNA sequences and open reading frames for mitochondrially encoded proteins. The EMBO Journal 13, 898-905.

KATOH, K., KUMA, K., TOH, H., MIYATA, T., 2005. MAFFT version 5: Improvement in accuracy of multiple sequence alignment. Nucleic Acids Research 33, 511-518.

LANDT, O., NITSCHE, A., 1999. Selection of hybridization probe sequences for use with the LightCycler. Roche Molecular Biochemicals: Technical Note No. LC 6/99, 1-8.

LIENARD, M. A., LASSANCE, J. M., PAULMIER, I., PICIMBON, J. F., LOFSTEDT, C., 2006. Differential expression of cytochrome c oxidase subunit III gene in castes of the termite reticulitermes santonensis. Journal of Insect Physiology 52, 551-557.

MANS, B.J., PIENAAR, R., LATIF, A.A., POTGIETER, F.T., 2011. Diversity in the 18S SSU rRNA V4 hyper-variable region of *Theileria* spp. in Cape buffalo (*Syncerus caffer*) and cattle from southern Africa. Parasitology 1-14. doi:10.1017/S0031182011000187

OP DEN BUIJSCH, R.A.M., DE VRIES, J.E., LOOTS, W.J.G., LANDT, O., WIJNEN, P.A.H.M., VAN DIEIJEN-VISSER, M.P., BEKERS, O., 2005. Genotyping of the PXR A11156C polymorphism with locked nucleic acid containing fluorogenic probes. The Pharmacogenomics Journal 5, 72-74.

PIENAAR, R., POTGIETER, F. T., LATIF, A. A., THEKISOE, O. M., MANS, B. J., 2011. Mixed *Theileria* infections in free-ranging buffalo herds: Implications for diagnosing *Theileria parva* infections in Cape buffalo (*Syncerus caffer*). Parasitology 1-12.

POSADA, D., CRANDALL, K.A., 1998. MODELTEST: testing and model of DNA substitution. Bioinformatics 14, 817-818.

REUTER, M., KUPPER, Y., SCHMITZ, A, BREUER, J.P., WEND, U., HENNIG, J., 2005. Detection of new single nucleotide polymorphisms by means of real time PCR. Indian Academy of Sciences 84, 341-345.

RONQUIST, F., HUELSENBECK, J.P., 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19, 1572-1574.

SCHMIDT, T. R., JARADAT, S. A., GOODMAN, M., LOMAX, M. I., GROSSMAN, L. I., 1997. Molecular evolution of cytochrome c oxidase: Rate variation among subunit VIa isoforms. Molecular Biology and Evolution 14, 595-601.

SIBEKO, K.P., OOSTHUIZEN, M.C., COLLINS, N.E., GEYSEN, D., RAMBRITCH, N.E., LATIF, A.A., GROENEVELD, H.T., POTGIETER, F.T., COETZER, J.A.W., 2008. Development and evaluation of a real-time polymerase chain reaction test for the detection of *Theileria parva* infections in Cape buffalo (*Syncerus caffer*) and cattle. Veterinary Parasitology 155, 37-48.

STADEN, R., BEAL, K. F., BONFIELD, J. K., 2000.The staden package, 1998. Methods in Molecular Biology 132, 115-130.

SWOFFORD, D.L., 2003. PAUP*.Phylogenetic Analysis Using Parsimony (*and other methods). Version 4b10. Sinauer Associates, Sunderland, Massachusetts.

TAMURA, K., DUDLEY, J., NEI, M., KUMAR, S., 2007. MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. Molecular Biology and Evolution 24, 1596-1599.

UILENBERG, G., 1999. Immunization against diseases caused by *Theileria parva*: A review. Tropical Medicine and International Health 4, A12-20.

WANG, C., AHLUWALIA, S.K., LI, Y., GAO, D., POUDEL, A., CHOWDHURY, E., BOUDREAUX, M.K., KALTENBOECK, B., 2010. Frequency and therapy monitoring of canine *Babesia* spp. infection by high-resolution melting curve quantitative FRET-PCR. Veterinary Parasitology 168, 11-18.

ZAHLER, M., SCHEIN, E., RINDER, H., GOTHE, R., 1998. Characteristic genotypes discriminate between *Babesia canis* isolates of differing vector specificity and pathogenicity to dogs. Parasitology Research 84, 544-548.

ZARLENGA, D. S., HIGGINS, J., 2001. PCR as a diagnostic and quantitative technique in veterinary parasitology. Veterinary Parasitology 101, 215-230.