CHAPTER 5

Sequence heterogeneity in the equi merozoite antigen gene (*ema-1*) of *Theileria equi* and development of an *ema-1*-specific TaqMan MGB™ assay for the detection of *T. equi*

5.1 Abstract

A *Theileria equi ema-1*-specific quantitative real-time PCR assay (qPCR) (Ueti et al., 2003) was tested on 107 South African field samples, 90 of which tested positive for *T. equi* antibody using the immuno-fluorescent antibody test (IFAT). The qPCR assay performed poorly, as *T. equi* was detected in only 67 of the 90 IFAT-positive field samples at quantification cycle (C_q) values ranging from 27 to 39.95. Furthermore, a high C_q value of 36.18 was obtained from DNA extracted from a South African *in vitro*-cultured *T. equi* WL isolate [1.38% parasitized erythrocytes (PE)] when a low C_q value (high *T. equi* concentration) was expected. Approximately 600 bp of the *ema-1* gene from 38 samples were sequenced and BLASTN analysis confirmed all sequences to be merozoite surface protein genes, with an identity of 87.1 - 100% to previously published *T. equi ema-1* gene sequences. Alignment of the sequences revealed extensive sequence variations in the target regions of the primers and probes (Ueti et al., 2003), explaining the poor performance of the qPCR assay. Based on these observations, we developed a new TaqMan minor groove binder (MGB™) probe-based qPCR assay, targeting a more conserved region of the *ema-1* gene. This assay was shown to be efficient and specific, and the detection limit, defined as the concentration at which 95% of *T. equi* positive samples are detected, was determined to be 0,00014% PE. The two *ema-1* assays were compared by testing 41 South African field samples in parallel. The results suggested that the new assay was more sensitive than the original assay, as *T. equi* was detected in more samples and at lower C_q values when the new assay was used. Phylogenetic analyses of the 18S rRNA gene sequences and *ema-1* amino acid sequences from the same samples showed inconsistencies between the clades, indicating that the *T. equi* 18S rRNA genetic groups identified in South Africa may not represent distinct *T. equi* lineages and that genetic recombination most probably occurs within *T. equi* parasite populations in South Africa, explaining the sequence diversity observed.

5.2 Introduction

The pathogenesis of haemoprotozoan diseases is orchestrated by merozoite surface proteins, which play pivotal roles in the recognition of, attachment to and penetration of host erythrocytes by parasites (Knowles et al., 1991a). The surface-exposed, immunodominant protein expressed during the *T. equi* merozoite stage, equi merozoite antigen-1 (EMA-1) is an important candidate for the development of effective diagnostic assays (Knowles et al., 1991a). Xuan et al. (2001) reported a high degree of homology between amino acid sequences of EMA-1 from 19 *T. equi* strains from various countries and orthologous gene sequences have been described for a number

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of other *Theileria* species (Katende et al., 1990; Kawazu et al., 1992; Matsuba et al., 1995; Shiels et al., 1995; d'Oliveira et al., 1996). A competitive-inhibition ELISA (cELISA) employing a monoclonal antibody (MAb 36/133.97) to recombinant EMA-1 has been shown to reliably detect antibody to *T. equi* in the sera of infected horses from 19 countries (Knowles et al., 1992; Shkap et al., 1998; Sevinc et al., 2008).

The recent application of molecular biological techniques has resulted in the improved detection, identification and genetic characterization of many haemoprotozoan parasites (Caccio et al., 2000; Nagore et al., 2004). The polymerase chain reaction (PCR) has been applied for the detection of many *Theileria* and *Babesia* species and has been reported to have higher sensitivities and specificities when compared to serological assays (Geysen et al., 2003; Buling et al., 2007; Jefferies et al., 2007). Many current molecular assays target nuclear rRNA genes specifically for species identification (e.g. Allsopp et al., 1993), and a number of such PCR tests have been designed to detect *T. equi* (Bashiruddin et al., 1999; Gubbels et al., 1999; Nagore et al., 2004). Many of these assays will not reliably detect all *T. equi* variants, as a previous study in our laboratory revealed extensive sequence variation in the 18S rRNA gene in South African *T. equi* parasites, where three distinct genetic groups were identified (Bhoora et al., 2009a; Chapter 2). Although conserved regions in the 18S rRNA gene exist, and a sensitive and specific qPCR assay for detection of *T. equi* has been developed and evaluated (Kim et al., 2008; Bhoora et al., 2009b; Chapter 3), the existence of as yet undetected 18S rRNA gene sequence variants cannot be ruled out. It would therefore be advantageous to develop a qPCR assay targeting a different gene, which could be used to confirm results obtained from the *T. equi* 18S rRNA qPCR assay.

Genes encoding outer membrane proteins have previously also been targeted for the development of molecular diagnostic assays (Nicolaiewsky et al., 2001; Ueti et al., 2003; Alhassan et al., 2005; Alhassan et al., 2007; Heim et al., 2007). Due to their surface location, however, they are likely to be under intense selection pressure, and sequence heterogeneity in the genes encoding these proteins is expected to occur (Allsopp and Allsopp, 2006). Preliminary studies in our laboratory revealed some discrepencies between the IFAT and cELISA, with the IFAT performing slightly better than the commercially available cELISA in the detection of *T. equi* antibody in the sera of infected horses (Bhoora et al., 2010; Chapter 6). Sequence differences in the EMA-1 antigen within South African *T. equi* isolates may have prevented the detection of *T. equi* antibodies by the cELISA assay in some samples.

Despite the possibility of heterogeneity within the *T. equi ema-1* gene, it has been used as a target in the development of a TaqMan qPCR assay (Ueti *ema-1* qPCR assay) for the quantification of parasite load in *Rhipicephalus* (*Boophilus*) *microplus* ticks, as well as in the peripheral blood of a splenectomized pony experimentally infected with the Florida strain of *T. equi* (Ueti et al., 2003). In this study, the Ueti *ema-1* qPCR assay was tested on South African field samples, and following the poor performance of the test, the *ema-1* gene was sequenced to determine the extent of sequence heterogeneity. This was followed by the development of a sensitive TaqMan MGB™ qPCR assay targeting a more conserved region of the *ema-1* gene for the detection of *T. equi* infections in South African horses.

5.3 Materials and Methods

5.3.1 *Theileria equi field samples and isolates*

Serum and EDTA-treated blood samples were collected from 107 horses at the South African National Yearling Sale in 2006 (Bhoora et al., 2009a; Chapter 2), and from 41 horses of unknown piroplasm status, resident on three stud farms in the Northern Cape Province, South Africa (Bhoora et al., 2009b; Chapter 3 Section 3.3.3). Field samples from horse (LFEQ23, LFEQ47, LFEQ189) and zebra (RBEQ101 and RBEQ96), for which the 18S rRNA gene had previously been characterized (Bhoora et al., 2009a; Chapter 2), were also used in this study. The *T. equi* WL isolate (Bhoora et al., 2009b; Chapter 3) was cultured as described previously (Zweygarth et al., 1995).

5.3.2 *Serological assays*

Sera from the 107 National Yearling Sale horse samples were tested for *T. equi* antibody, using (i) a standard IFAT protocol, as described in the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (Anonymous, 2008), with the exception that *in vitro*-cultured antigen produced locally in South Africa was used, and (ii) a commercially available cELISA kit as described by the manufacturer (VMRD Inc., Pullman, WA, USA). The IFAT was performed at the Agricultural Research Council-Onderstepoort Veterinary Institute (ARC-OVI) and the cELISA was performed at the Department of Veterinary Tropical Diseases.

5.3.3 *In vitro culture diagnosis*

A modified HL-1 medium (BioWhittaker, Walkersville, MD, USA) (Holman et al., 1994), was used for the *in vitro* culture of *T. equi* from blood samples as described previously (Zweygarth et al., 1995). The medium was supplemented with 20% horse serum, 2 mM L-glutamine, 0.2 mM hypoxanthine, 1 mM L-cysteine hydrochloride, 0.02 mM 2,9-dimethyl-4,7-diphenyl-1,10-phenanthrolinedisulphonic acid disodium salt (bathocuproine sulphonate, BCS; Serva Feinbiochemica, Heidelberg, Germany), 100 IU/ml penicillin and 100 µg/ml streptomycin. The medium was buffered with 15 mM HEPES and 2.2 g l^{-1} $NaHCO_3$.

A piroplasm-free mare, which was confirmed seronegative for *T. equi* and *B. caballi* using IFAT, was used to collect horse red blood cells (HRBC) by venipuncture into sterile Vac-u-test$^®$ tubes containing EDTA as anticoagulant. They were washed four times by centrifugation (650 x g, 10 min, room temperature) and resuspended in a modified Vega y Martinez phosphate-buffered saline solution (Vega et al., 1985) omitting adenine, guanosine and the antibiotics (mVYM). After each wash, the white blood cell layer overlaying the HRBC was removed. After the fourth and final wash, the HRBC were resuspended in mVYM solution and stored at 4 °C until used.

For culture diagnosis, erythrocytes from each test sample were prepared as described above and 100 µl erythrocyte suspension was resuspended in 900 µl complete culture medium and distributed into 24‑well culture plates. The plates were incubated at 37° C in either a humidified 5% CO_2-in-air atmosphere or a humidified gas mixture of 5% CO_2, 2% O_2 and 93% N_2. Medium was changed daily by replacement of 700 µl of medium overlaying the erythrocytes in each well. After 5 days, the plate was transferred to an incubator with 5% CO_2 in air.

The *in vitro* culture diagnosis of *T. equi* was performed at the ARC-OVI.

5.3.4 *DNA extraction*

Genomic DNA was extracted from 200 µl of EDTA-treated blood or 200 µl of *in vitro* culture material using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

5.3.5 *Theileria equi*-specific qPCR assays

The Ueti *ema-1* qPCR assay (primers and probes shown in Table 5.1) was used to detect *T. equi* infections in DNA extracted from 148 equine blood samples (n=107, National Yearling Sale 2006 and n=41, field samples, Northern Cape Province, South Africa). qPCR assays were performed in MicroAmp optical 96-well reaction plates using the StepOnePlus™ Real-time PCR instrument (v. 2.0, Applied Biosystems). Reactions were performed in a total volume of 20 µl comprising 1 x TaqMan Fast Universal PCR Master Mix (Applied Biosystems), 0.9 µM of each forward and reverse primer, 0.25 µM TaqMan probe and 5 µl of genomic DNA. The qPCR cycling conditions were as follows: activation of the FastStart DNA polymerase at 95 ºC for 20 s, then 40 cycles of 1 s at 95 ºC and 20 s at 60 ºC.

Primers and probes (Table 5.1), previously designed for a *T. equi*-specific qPCR assay based on the amplification of a region of the 18S rRNA gene (Kim 18S qPCR assay) (Kim et al., 2008), were used to detect *T. equi* infections in DNA extracted from all 148 equine blood samples. qPCR was performed as described above.

Table 5.1 Oligonucleotide primer and probe sequences used in the *T. equi*-specific qPCR assays.

Primer/Probe	Sequence 5'- 3'	Reference
ema1_f	GAGTCCATTGACCACGTCACC	(Ueti et al., 2003)
ema1_r	GTGCCTGACGACAGTCTTTGG	(Ueti et al., 2003)
ema1_probe	6-FAM-TCGACAAGCAGTCCGAGGAGCACA-TAM	(Ueti et al., 2003)
Be18SF	GCGGTGTTTCGGTGATTCATA	(Kim et al., 2008)
Be18SR	TGATAGGTCAGAAACTTGAATGATACATC	(Kim et al., 2008)
Be18SP	6-VIC-AAATTAGCGAATCGCATGGCTT	(Kim et al., 2008)
RT_EMAF	CCGGCAAGAAGCACAY*CTT	This study
RT_EMAR	TGCCATCGACGAYCTTGAG	This study
RT_EMAprobe	6-FAM-TCCAGGCAAGCGC-MGB	This study

*Ambiguity code : Y=C/T

5.3.6   Amplification and sequencing

Published *T. equi ema-1* gene sequences [*B. equi* isolate 212, AB015212; *B. equi* isolate H-25, AB015208, *B. equi* USDA strain, AB043618 (Xuan et al., 2001); *B. equi* Russia, AB015211; *B. equi* isolate E12, AF261824; *B. equi* Brazil, U97167 (Knowles et al., 1997); *B. equi* Florida, L13784 (Kappmeyer et al., 1993); *B. equi* Morocco, U97168 (Knowles et al., 1997)], were used to design gene-specific primers, TE-EMA1F (5'-TCC ATT GCC ATT TCG AGC ATC CT-3') and TE-EMA1R (5'-TTG ATT CTG CCA TCG CCC TTG-3') for the amplification of a 602 bp fragment of the *T. equi ema-1* gene from South African field samples and isolates.   The Genomiphi DNA amplification kit (Amersham Biosciences) was used to exponentially amplify genomic DNA from samples with low parasitaemias, prior to performing the PCR.   Reactions were performed in a final volume of 25 µl with High Fidelity PCR Master Mix (Roche Diagnostics, Mannheim, Germany), 0.2 µM of each primer and 2.5 µl amplified genomic DNA. The cycling conditions were: an initial denaturation of 5 min at 95 ºC, followed by 35 cycles of 30 s at 95 ºC, 1 min at 60 ºC and 1 min at 72 ºC, and a final extension of 7 min at 72 ºC.

The V4 hypervariable region of the 18S rRNA gene was amplified using a nested PCR as described previously (Bhoora et al., 2009a; Chapter 2).   Briefly, primary PCRs were performed using primers NBabesia1F (5'-AAG CCA TGC ATG TCT AAG TAT AAG CTT TT-3') and 18SRev-TB (5'-GAA TAA TTC ACC GGA TCA CTC G-3'), and 2.5 µl of genomic DNA that had been amplified using the Genomiphi DNA amplification kit.   Primers BT18S2F (5'-GGG TTC GAT TCC GGA GAG GG-3') and BT18S2R (5'-CCC GTG TTG AGT CAA ATT AAG CCG-3') were used to amplify the V4 hypervariable region from primary PCR products (Bhoora et al., 2009a; Chapter 2).

PCR products were purified using the Qiagen PCR purification kit (Qiagen, Hilden, Germany) and sequenced using BigDye chemistry (v.3.1, Applied Biosystems) on a 3130XL genetic analyzer (Applied Biosystems).   Sequences were assembled and edited using the Staden software suite (Staden, 1996).

5.3.7 Phylogenetic analyses

Multiple sequence alignments were performed using MAFFT employing the FFT-NS-1 algorithm (Katoh et al., 2005) and alignments were adjusted manually using BioEdit, version 7.0.5.2 (Hall, 1999). Aligned *ema-1* nucleotide sequences were translated into amino acid sequences using the ExPasy translate tool (www.expasy.ch/tools/dna). Modeltest, v.3.7 (Posada and Crandall, 1998), was used to identify a TRN + I + G substitution model with a proportion of invariable sites and a gamma-shaped distribution of rates across sites. PAUP* v4b10 (Swofford, 2003) was used to carry out the phylogenetic analyses of the 18S rRNA nucleotide sequences and *ema-1* nucleotide and amino acid sequences using distance, parsimony and maximum-likelihood methods. The EMA-1 phylogenetic tree was constructed (1000 replicates) using the merozoite surface amino acid sequence of *Plasmodium falciparum* (AAX55745) as an outgroup, while the 18S rRNA gene sequence of the *Hepatozoon canis* isolate Venezuela 1 (DQ439543) was used as an outgroup in the construction of the 18S rRNA phylogenetic tree. Analysis by Bayesian inference was performed using MrBayes v.3.1.2 (Ronquist and Huelsenbeck, 2003), accessible via the Computational Biology Service Unit at Cornell University (http://cbsuapps.tc.cornell.edu/mrbayes.aspx). Four simultaneous Markov chain Monte Carlo (MCMC) chains were run for 5,000,000 generations, with sampling every 100 generations for a total of 50,000 samples per run. All consensus trees generated were edited using MEGA4 (Tamura et al., 2007).

5.3.8 Theileria equi ema-1-specific TaqMan MGB™ qPCR assay

An alignment of *ema-1* gene sequences obtained from 19 South African samples and 8 published *T. equi ema-1* sequences available on Genbank (Accession numbers: AB015211, AF261824, U97167, U97168, L13784, AB043618, AB015208 and AB015212) was used to identify a more conserved region of the *ema-1* gene for the development of an *ema-1*-specific TaqMan minor-groove binder (MGB™) probe qPCR assay (Bhoora *ema-1*qPCR assay). The Primer express software v3.0 (Applied Biosystems) was used to design the qPCR primers and probes. A primer pair, RT-EMA1F and RT-EMA1R (Integrated DNA Technologies, IDT), in combination with a TaqMan MGB™ probe, RT-EMAprobe (Applied Biosystems), was designed to amplify and detect a 59 bp fragment of the *ema-1* gene (Table 5.1) using the StepOnePlus™ Real-time PCR instrument (Applied Biosystems). Reactions were performed as described above.

A ten-fold dilution series of the *in vitro*-cultured South African *T. equi* WL isolate, with a PE of approximately 13.8% (1.24×10^6 parasites/µl), was used to generate a standard curve from which the efficiency of the *T. equi ema-1* qPCR assay was determined. The dilution series (10^0 to 10^{-6}) was prepared in duplicate using uninfected equine red blood cells. DNA was extracted from 200 µl of all diluted samples and qPCR amplifications of the standard dilution series were repeated in triplicate and on ten separate days. The data generated from each of the 30 runs were used to calculate a linear regression equation of C_q (Bustin et al., 2009) against log copy number, from which the efficiency of the assay was determined.

SigmaPlot[®] (ver. 11) was used to plot a sigmoidal regression equation of sensitivity against log dilution, from which the sensitivity of the assay was determined. The estimated sensitivity and 95% confidence intervals for the true sensitivity of the dilution series prepared, was calculated using the standard error of the estimated sensitivity of the dilution series (Sibeko et al., 2008). The analytical specificity of the assay was evaluated by using DNA extracted from other protozoal parasites expected to occur in equids, including *Trypanosoma brucei evansi*, *Trypanosoma vivax*, *Trypanosoma brucei equiperdum* and *B. caballi*. DNA extracted from a piroplasm-free horse, confirmed negative by IFAT, was included in the assay as a negative control.

5.3.9   *Evaluation of the T. equi qPCR assays*

Forty-one serum and EDTA-treated blood samples, of unknown piroplasm status, were collected from horses resident on three stud farms in the Northern Cape Province, South Africa, where tick-control measures were only implemented when high tick burdens were observed. Sera were tested for the presence of antibody against *T. equi* using the IFAT (Bhoora et al., 2009b; Chapter 3 Table 3.3). DNA extracted from EDTA-treated blood samples was tested for the presence of *T. equi* parasites using the Kim 18S, Ueti *ema-1* and Bhoora *ema-1* qPCR assays.

5.4   Results

Of the 107 samples collected from horses at the National Yearling Sale (2006), the IFAT and cELISA detected *T. equi* antibodies in 90 and 88 samples, respectively (Appendix B). The *in vitro* culture technique detected *T. equi* in 90 samples. The Kim 18S qPCR assay detected *T. equi* in 92 samples, while the Ueti *ema-1* qPCR assay detected *T. equi* in only 67 samples. All five tests were in agreement for 74 samples (69.2%, 61 positive, 13 negative), although some

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samples were detected by the Ueti *ema-1* qPCR assay at much higher C_q values than those obtained with the Kim 18S qPCR assay. Twenty samples that were *T. equi* positive using IFAT, cELISA, *in vitro* culture and the Kim 18S qPCR assay, tested negative using the Ueti *ema-1* qPCR assay. Conflicting results were obtained for the remaining 13 samples, which were positive by at least one test (Table 5.2). In addition, the Ueti *ema-1* qPCR assay detected DNA extracted from a South African *in vitro*-cultured *T. equi* isolate (1.38% PE) at an unexpectedly high C_q value of 36.18. In comparison, this sample tested positive at a C_q value of 22.7 using the Kim 18S qPCR assay.

Table 5.2 Conflicting test results for *T. equi* from 13 samples obtained from horses at the National Yearling Sale 2006, South Africa.

Sample	IFAT	cELISA	*In vitro* culture	Kim 18S rRNA qPCR assay (Kim et al., 2008)	Ueti *ema-1* qPCR assay (Ueti et al., 2003)
EQ2	+	+	+	-	-
EQ30	-	+	+	29.42	29.22
EQ33	-	-	+	31.51	33.9
EQ35	-	+	+	28.30	31.19
EQ50	+	-	+	31.21	-
EQ54	+	-	+	32.04	-
EQ63	+	+	+	-	36.52
EQ70	+	+	-	32.11	-
EQ71	+	+	-	35.15	-
EQ81	+	+	-	32.55	34.83
EQ94	+	-	+	31.25	33.22
EQ103	+	+	+	-	-
EQ107	-	-	-	37.45	-

A ~600 bp fragment of the *ema-1* gene was amplified and sequenced from the *in vitro*-cultured South African *T. equi* WL isolate and from 18 field samples. Sequences were obtained from 10 of the 20 *T. equi*-positive samples that were not detected using the Ueti *ema-1* qPCR assay (EQ6, EQ25, EQ37, EQ38, EQ43, EQ44, EQ46, EQ51, EQ59 and EQ67), from three samples that were detected by the Ueti *ema-1* qPCR assay at C_q values between 36.93 and 39.34 (EQ17, EQ47 and EQ74), and from five South African horse and zebra samples, for which the 18S rRNA gene had previously been characterized (LFEQ23, LFEQ47, RBEQ101, LFEQ189 and RBEQ96) (Bhoora et al., 2009a; Chapter 2). BLAST analysis confirmed that all sequences obtained were merozoite surface protein genes, which showed between 87.5 and 100% identity to previously published

T. equi ema-1 gene sequences (Accession numbers: AB015211, AF261824, U97167, U97168, L13784, AB043618, AB015208 and AB015212).

Phylogenetic analyses of both the *ema-1* nucleotide sequences and the predicted EMA-1 amino acid sequences yielded trees with almost identical topologies and high bootstrap or nodal support values. The analyses showed that the EMA-1 amino acid sequences obtained in this study fell into three main groups, designated A, B and C (Figure 5.1). Group A contained two EMA-1 sequences from samples for which the 18S rRNA gene sequence had previously been determined [LFEQ23 and LFEQ47 (Bhoora et al. 2009a; Chapter 2)]; these grouped together with a number of EMA-1 amino acid sequences from *T. equi* isolates from Brazil, Morocco, Florida and Russia (Kappmeyer et al., 1993; Knowles et al., 1997; Xuan et al., 2001) (Accession numbers, respectively: AAC38826, AAC38827, AAA72370, BAA32978) (Figure 5.1). Group B contained the *T. equi* USDA strain (Accession number: BAA96134), and four South African EMA-1 sequences. Two of these were obtained from samples for which the 18S rRNA gene had previously been characterized [RBEQ101 and RBEQ96 (Bhoora et al., 2009a; Chapter 2)], while two were obtained from samples that were detected by the Ueti *ema-1* qPCR assay at very high C_q values (EQ17 and EQ47). The remaining 13 South African EMA-1 sequences grouped together with two EMA-1 sequences from isolates 212 and H-25 (Accession numbers: BAA32979 and BAA32975) in group C. Ten of these South African sequences were from samples that were not detected by the Ueti *ema-1* qPCR assay, while two were from samples that were detected by the Ueti *ema-1* qPCR assay at very high C_q values (EQ74 and the *in vitro*-cultured South African *T. equi* WL isolate). The remaining sequence in group C was obtained from a sample for which the 18S rRNA gene had previously been characterized [LFEQ189 (Bhoora et al., 2009a; Chapter 2)].
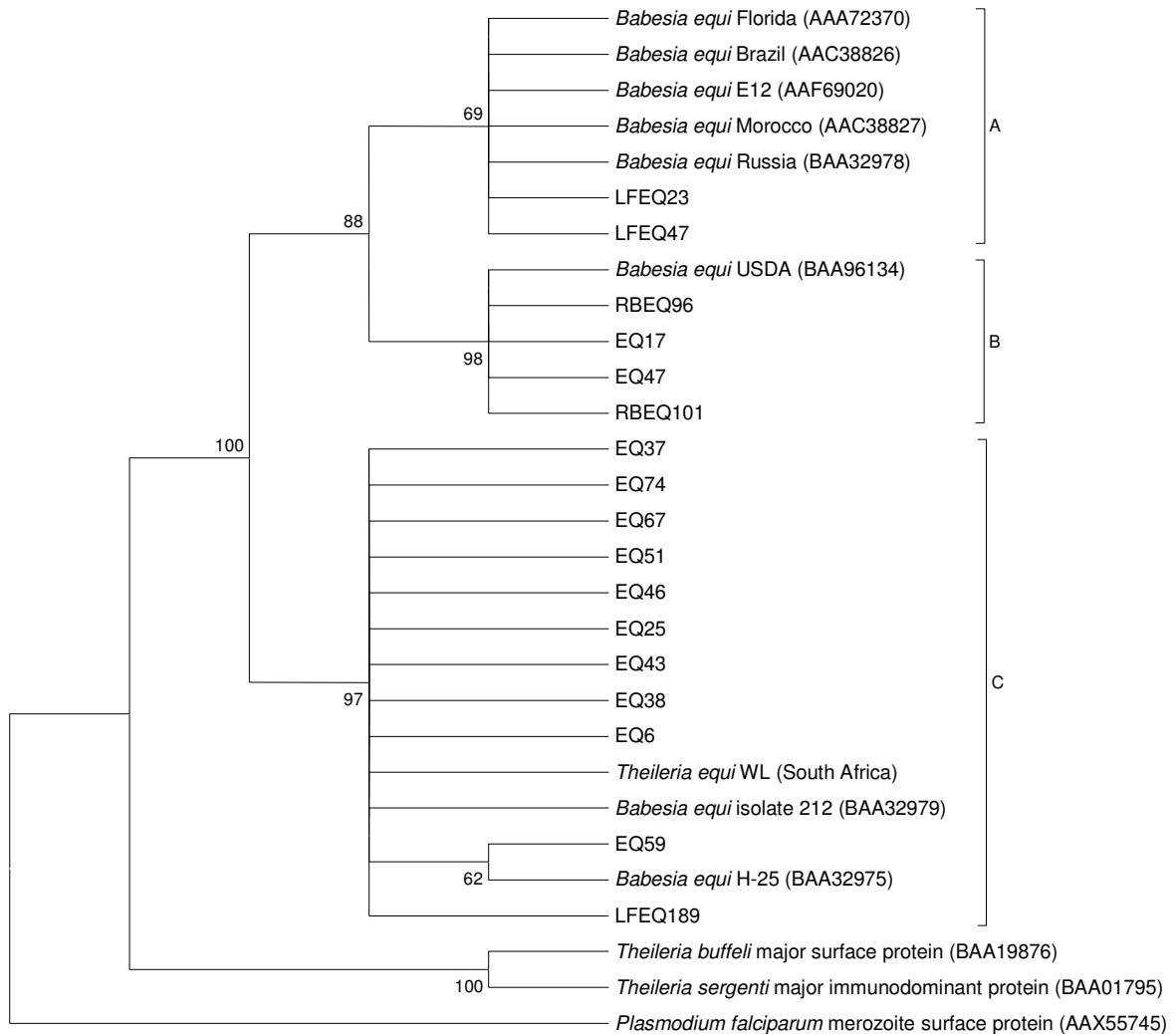
Figure 5.1 Rooted 50% majority-rule consensus cladogram, calculated using maximum-parsimony and bootstrap estimates from 1000 replicates of the EMA-1 amino acid sequences identified in this study and the *T. equi* EMA-1 sequences from the public sequence database (accession numbers are indicated in parenthesis). Bootstrap values are indicated on the nodes of the tree.
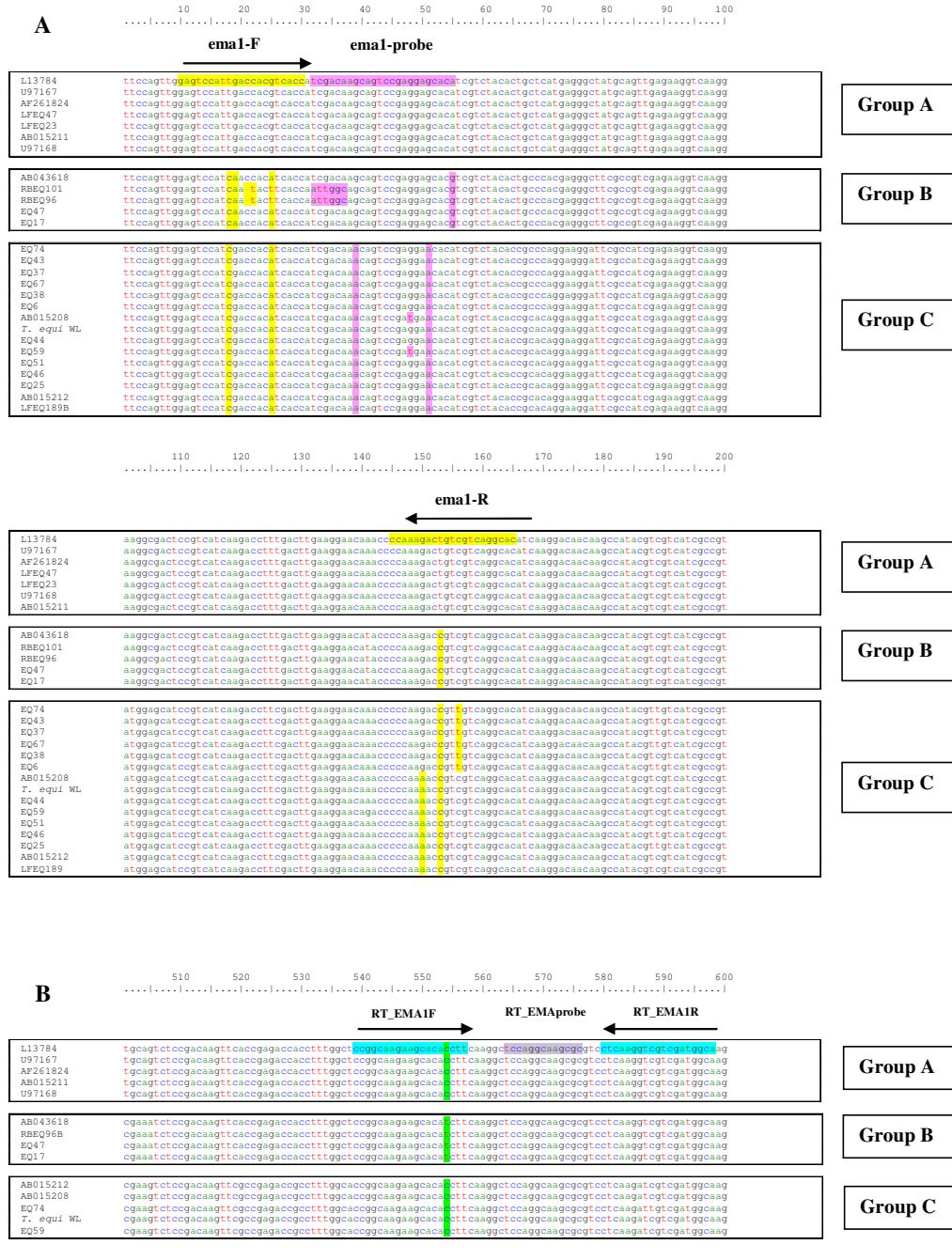
Figure 5.2   **(A)** Nucleotide alignment of *T. equi ema-1* gene sequences obtained in this study, to published *T. equi ema-1* gene sequences (*B. equi* isolate 212, AB015212; *B. equi* isolate H-25, AB015208, *B. equi* USDA strain, AB043618; *B. equi* Russia, AB015211; *B. equi* isolate E12, AF261824; *B. equi* Brazil, U97167; *B. equi* Florida, L13784; *B. equi* Morocco, U97168). The sequences of the real-time forward (ema1-F) and reverse (ema1-R) primers designed by Ueti et al. (2003) are highlighted in yellow. The probe sequence (ema1-probe) is highlighted in pink. Nucleotide differences in the primer and probe regions are highlighted in the same colors used to indicate the oligonucleotides. **(B)** An alignment of the *T. equi ema-1* gene sequences indicating the region where the Bhoora *ema-1* qPCR primers and probes were designed. The Bhoora *ema-1* qPCR primers (RT_EMA1F and RT_EMA1R) are highlighted in blue and the probe sequence (RT_EMAprobe), is highlighted in purple. Nucleotides highlighted in green indicate the position of primer degeneracy. Groups A, B and C represent the three *T. equi* 18S rRNA groups identified in this study.

Sequence alignments demonstrated the occurrence of nucleotide variation in the regions of the *ema-1* gene that the Ueti qPCR primers and probes had targeted (Figure 5.2A). Sequences in group A, which contained the Florida *ema-1* sequence (Accession number: L13784) used by Ueti et al. (2003) to design the qPCR test, showed no nucleotide heterogeneity in the regions where the primers and probe had been designed. Three sequences occurring in group B, including the *ema-1* gene sequence from the *B. equi* USDA strain (accession number AB043618), contained three nucleotide differences in the forward primer sequence, a single nucleotide difference in the reverse primer sequence and one difference at the 3' end of the qPCR probe sequence. Group B also contained two *ema-1* sequences amplified from zebra samples (RBEQ101 and RBEQ96), which showed five nucleotide differences in the forward primer region, seven differences in the probe region and one difference in the reverse primer sequence. Similarly, for *ema-1* sequences in group C, two nucleotide differences occurred in the forward and reverse primer regions, while up to three differences could be observed in the qPCR probe region.

The Bhoora *ema-1* qPCR primers and probe were designed in a conserved region of the *T. equi ema-1* gene (Figure 5.2B). The forward primer (RT_EMA1F) was designed to contain a single nucleotide degeneracy, which allows for the detection all sequences in each of the three *ema-1* groups identified. The Bhoora *ema-1* qPCR assay had an efficiency of 93.8% in amplifying a 59 bp conserved region of the *ema-1* gene from the *in vitro*-cultured *T. equi* WL isolate (Figure 5.3A and B). *Theileria equi* DNA was detected in all 30 replicates of the dilutions of the *in vitro*-cultured *T. equi* WL isolate ranging from 1.38 to 1.38 x 10^{-3}% PE. At lower PE values, sensitivity decreased (Figure 5.3C). The detection limit of the assay, defined as the concentration at which 95% of the positive samples were detected, was 1.4 x 10^{-4}% PE at a C_q of 35.4. The assay was specific for *T. equi*, in that no amplification signals were observed from DNA extracted from any of the other protozoal parasites expected to occur in equids including *Babesia caballi* as well as a number of *Trypanosoma* species (results not shown).

The Bhoora *ema-1* qPCR assay detected *T. equi* DNA in 90 (84%) of the 107 previously tested samples at C_q values ranging between 24.39 and 34.70. The IFAT, cELISA, *in vitro* culture, Kim 18S qPCR and Bhoora *ema-1* qPCR assays were in full agreement for 94 samples (87.9%, 81 positive, 13 negative). The Bhoora *ema-1* qPCR assay detected *T. equi* DNA in all 20 samples that tested negative using the Ueti *ema-1* qPCR assay, but which were *T. equi*-positive using the four other assays (IFAT, cELISA, *in vitro* culture and *T. equi* 18S rRNA qPCR assay); the C_q values obtained were comparable to those obtained using the Kim 18S qPCR assay (Table

5.3). In addition, the Bhoora *ema-1* qPCR assay detected *T. equi* DNA in the South African *in vitro*-cultured *T. equi* WL isolate at a C_q value of 20.0, in contrast to the C_q value of 36.18 obtained from using the Ueti *ema-1* qPCR assay (Figure 5.4).
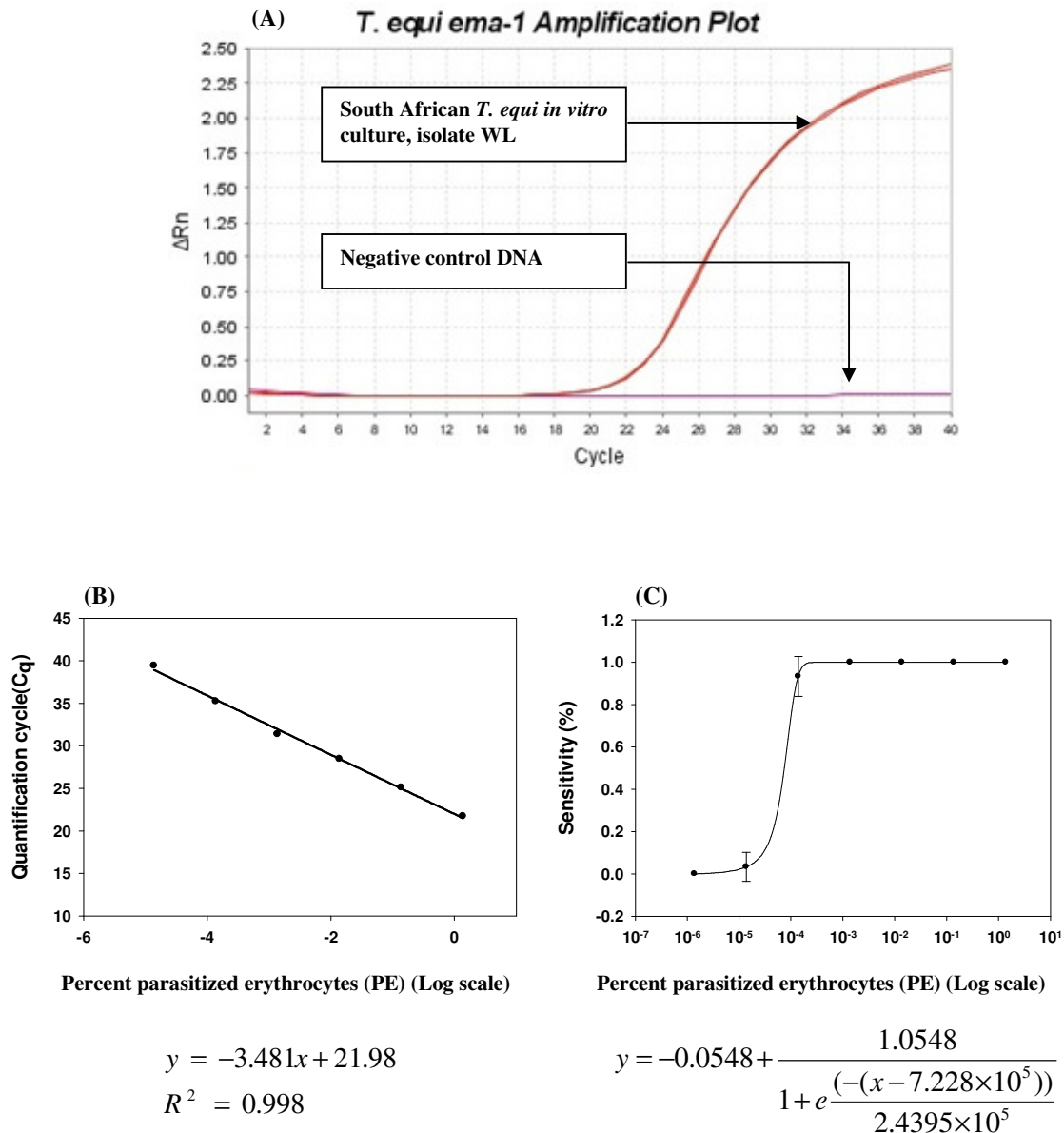

$$y = -3.481x + 21.98$$
$$R^2 = 0.998$$

$$y = -0.0548 + \frac{1.0548}{1 + e^{\frac{(-(x - 7.228 \times 10^5))}{2.4395 \times 10^5}}}$$

Figure 5.3 **(A)** Detection of positive control DNA (*T. equi* WL *in vitro*-cultured isolate) using the Bhoora *ema-1* qPCR assay, indicated by an increase in the fluorescence signal. No increase in fluorescence was observed in the negative control sample (DNA extracted from blood from a certified piroplasm free horse). The threshold (ΔRn) value was set at 0.03. **(B)** Standard curve for the quantification of the *T. equi ema-1* gene. C_q values are plotted against the log% parasitaemia of the initial 10-fold dilution series of the parasite DNA equivalent to 1.242×10^5 to 1.242 parasites/μl of infected blood. **(C)** The sensitivity and 95% confidence intervals for the *ema-1* TaqMan MGB™ qPCR assay. The sensitivity was determined using a 10-fold dilution series from 10^{-1} to 10^{-7} prepared from an *in vitro* culture of *T. equi* WL with approximately 1.38% PE.
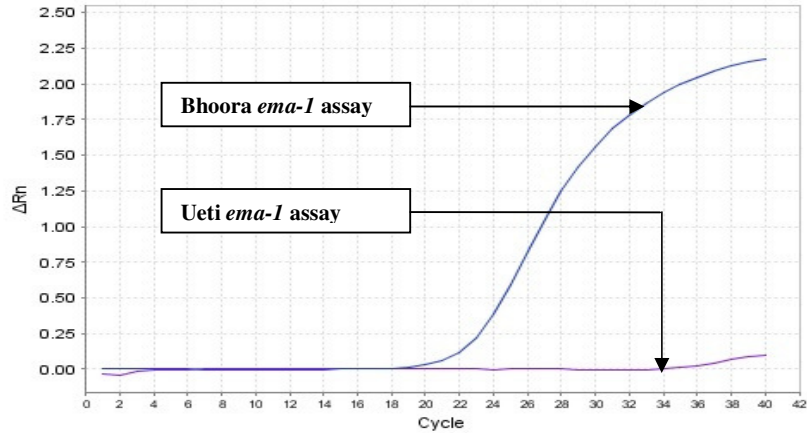
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Figure 5.4 Comparison of the efficiencies of the Ueti and Bhoora *ema-1* qPCR assays in the amplification of the *T. equi ema-1* gene from DNA extracted from the South African *T. equi* WL *in vitro*-cultured isolate with approximately 1.38% PE. The threshold (ΔRn) value was set at 0.03.

Forty-one field samples were subsequently used to evaluate the *T. equi* qPCR assays (Bhoora *ema-1*, Ueti *ema-1* and Kim 18S) (Table 5.4). The three tests were in full agreement for 36 samples (87.8%, 32 positive, 4 negative). The Bhoora *ema-1* qPCR assay detected *T. equi* in all samples that were IFAT-positive, whereas the other two qPCR assays failed to detect *T. equi* in two IFAT-positive samples (Kim11 and Kim20). In addition, the Bhoora *ema-1* qPCR assay detected *T. equi* in three samples that were IFAT-negative (Kim18, Kim31 and Kim40); the other two qPCR assays detected *T. equi* DNA in only one of these samples (Kim18).

Although *T. equi* could be detected in most of the 41 field samples using both *ema-1* qPCR assays, the C_q values obtained using the Bhoora *ema-1* qPCR assay were consistently lower than those obtained using the Ueti *ema-1* qPCR assay (Table 5.4). Furthermore, in seven samples (Kim2, Kim6, Kim14, Kim22, Kim28, Kim29 and Kim38), the Bhoora *ema-1* qPCR assay detected *T. equi* DNA at C_q values that were considerably lower than those obtained using the Ueti *ema-1* qPCR assay.

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Table 5.3 Comparison of test results for 20 samples obtained from horses at the National Yearling Sale 2006, South Africa, that tested negative for *T. equi* using the Ueti *ema-1* qPCR assay (Ueti et al., 2003), and tested positive using five other assays (Kim 18S qPCR assay, Bhoora *ema-1* qPCR assay, IFAT, cELISA and *in vitro* culture).

Sample	Ueti *ema-1* qPCR (Ueti et al., 2003)	Kim 18S qPCR assay (Kim et al., 2008)	Bhoora *ema-1* qPCR (this study)	IFAT	cELISA	*In vitro* culture
EQ6	-	28.85	26.88	+	+	+
EQ8	-	28.55	28.76	+	+	+
EQ10	-	29.40	29.56	+	+	+
EQ20	-	32.40	31.23	+	+	+
EQ25	-	28.58	25.92	+	+	+
EQ36	-	31.14	28.94	+	+	+
EQ37	-	31.54	29.94	+	+	+
EQ38	-	33.34	33.30	+	+	+
EQ43	-	31.96	27.76	+	+	+
EQ44	-	29.05	26.72	+	+	+
EQ46	-	28.99	26.32	+	+	+
EQ51	-	29.48	26.73	+	+	+
EQ53	-	31.05	28.92	+	+	+
EQ59	-	29.98	27.40	+	+	+
EQ60	-	32.85	31.01	+	+	+
EQ61	-	34.93	33.76	+	+	+
EQ64	-	30.93	28.62	+	+	+
EQ67	-	32.79	31.78	+	+	+
EQ79	-	32.52	30.35	+	+	+
EQ87	-	32.91	30.77	+	+	+

Phylogenetic analyses of the *ema-1* gene fragment amplified from 17 of the 41 field samples confirmed that the *ema-1* sequences fell within the same three groups described above (results not shown). Eleven sequences (Kim4, Kim10, Kim12, Kim16, Kim18, Kim23, Kim24, Kim25, Kim27, Kim32 and Kim41), which were all detected by the Ueti *ema-1* qPCR assay, clustered within group A. Four sequences (Kim6, Kim14, Kim20 and Kim38) clustered within group B, while the remaining two sequences (Kim28 and Kim30) occurred in group C. With the exception of Kim20 which was negative, the samples occurring in group B and C were detected using the Ueti *ema-1* qPCR assay, but at C_q values that were much higher than those obtained using the Bhoora *ema-1* qPCR assay.

Table 5.4 Comparison of test results from 41 field samples obtained from horses resident in the Northern Cape Province, South Africa.

Sample	IFAT	Ueti *ema-1* qPCR assay (Ueti et al., 2003)	*Bhoora ema-1* qPCR assay (this study)	Kim 18S qPCR assay (Kim et al. 2008)
Kim1	+	25.75	22.40	25.07
Kim2	+	30.43	22.30	25.14
Kim3	+	36.13	31.45	34.49
Kim4	+	24.28	21.16	24.18
Kim5	+	29.88	26.08	30.00
Kim6	+	31.40	23.94	25.53
Kim7	+	28.52	24.17	25.07
Kim8	+	33.93	30.85	34.13
Kim9	+	24.21	20.75	23.86
Kim10	+	24.78	21.81	24.19
Kim11	+	-	30.60	-
Kim12	+	22.16	18.87	22.37
Kim13	+	28.02	22.69	25.10
Kim14	+	30.38	21.53	24.09
Kim15	+	25.97	20.87	24.72
Kim16	+	25.86	22.84	26.04
Kim17	+	27.47	23.32	26.18
Kim18	-	28.85	27.02	30.13
Kim19	+	28.52	22.99	25.40
Kim20	+	-	25.61	-
Kim21	+	25.62	20.75	23.24
Kim22	+	32.61	22.21	25.03
Kim23	+	29.40	26.90	30.00
Kim24	+	22.15	19.41	22.88
Kim25	+	23.89	21.68	24.50
Kim26	+	33.09	30.27	32.16
Kim27	+	21.76	19.39	22.48
Kim28	+	34.23	19.75	22.86
Kim29	+	34.98	19.34	31.06
Kim30	+	31.58	28.21	23.17
Kim31	-	-	34.51	-
Kim32	+	29.25	26.14	29.09
Kim33	-	-	-	-
Kim34	-	-	-	-
Kim35	-	-	-	-
Kim36	-	-	-	-
Kim37	+	29.00	26.84	30.10
Kim38	+	37.33	27.67	30.44
Kim39	+	27.50	24.66	28.64
Kim40	-	-	35.40	-
Kim41	+	34.12	31.16	34.12

To determine whether the three *ema-1* groups identified in this study corresponded to the three 18S rRNA clusters identified previously (Bhoora et al., 2009a; Chapter 2), the V4 hypervariable region of the 18S rRNA gene was amplified and sequenced from 13 randomly selected field samples, representative of each EMA-1 phylogenetic group. The 18S rRNA sequence was also obtained from the *in vitro*-cultured South African *T. equi* WL isolate. Five 18S rRNA gene sequences determined previously [Accession numbers: EU888906, EU642510, EU888903, EU642507 and an unpublished sequence from RBEQ96 (Bhoora et al., 2009a; Chapter 2)] were included in the analysis from samples for which the *ema-1* gene was sequenced in this study (LFEQ23, LFEQ47, LFEQ189, RBEQ101 and RBEQ96, respectively). The 18S rRNA sequences from eleven of the samples clustered in group A, two in group B and six in group C (Figure 5.5). Of the eleven samples that clustered in 18S rRNA group A, only three samples (*T. equi* LFEQ23, Kim27 and Kim32) grouped in EMA-1 group A, two samples (Kim38 and EQ47) occurred in EMA-1 group B while the remaining six samples (*T. equi* WL, EQ38, EQ44, EQ25, EQ43 and Kim30) were found in EMA-1 group C (Figure 5.6). Both samples that were present in 18S rRNA group B (RBEQ101 and RBEQ96) also clustered in EMA-1 group B (Figure 5.6). Of the six samples that grouped in 18S rRNA group C, three (LFEQ47, Kim4 and Kim11) were found in EMA-1 group A, one (Kim20) in EMA-1 group B, and two (*T. equi* LFEQ189 and EQ6) in EMA-1 group C (Figure 5.6).
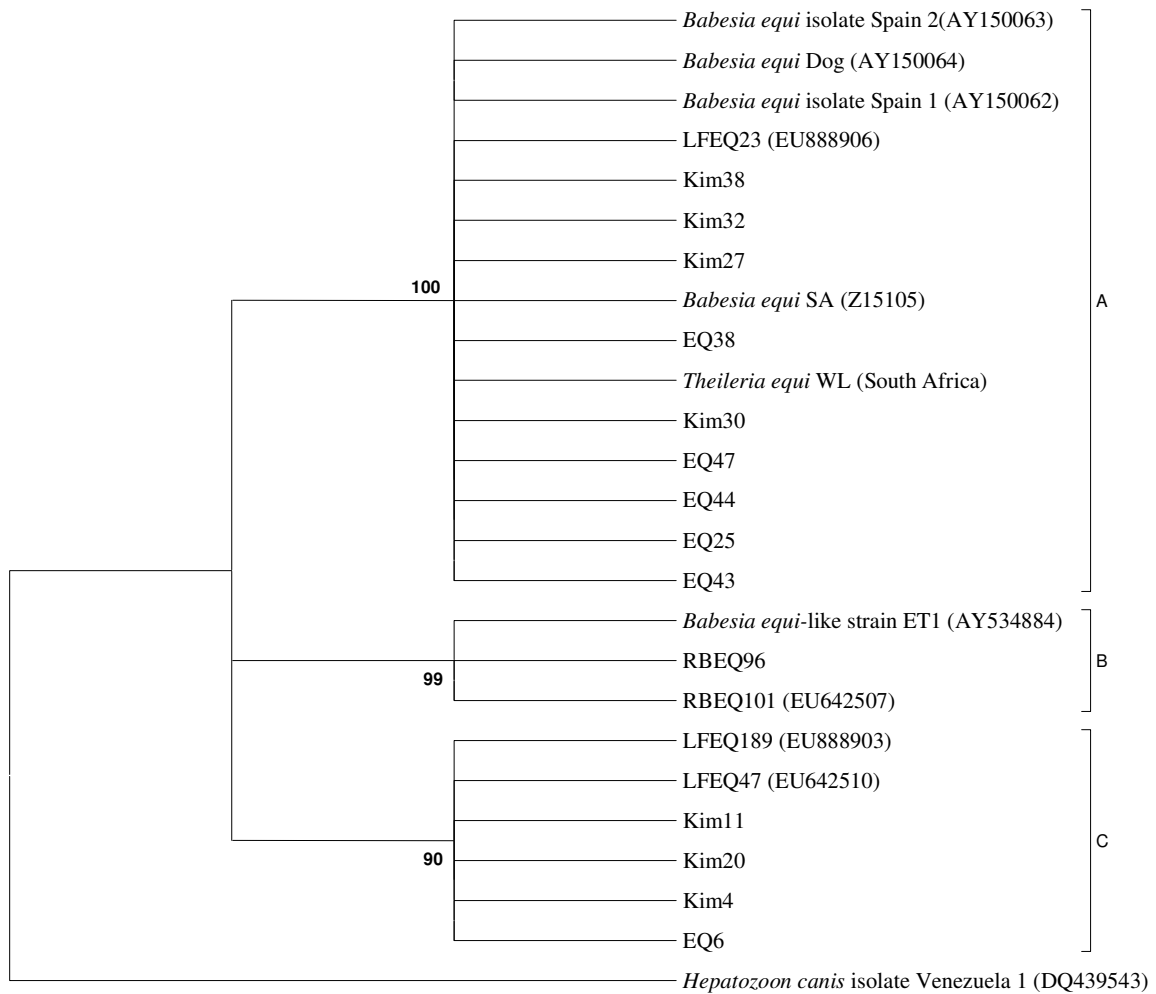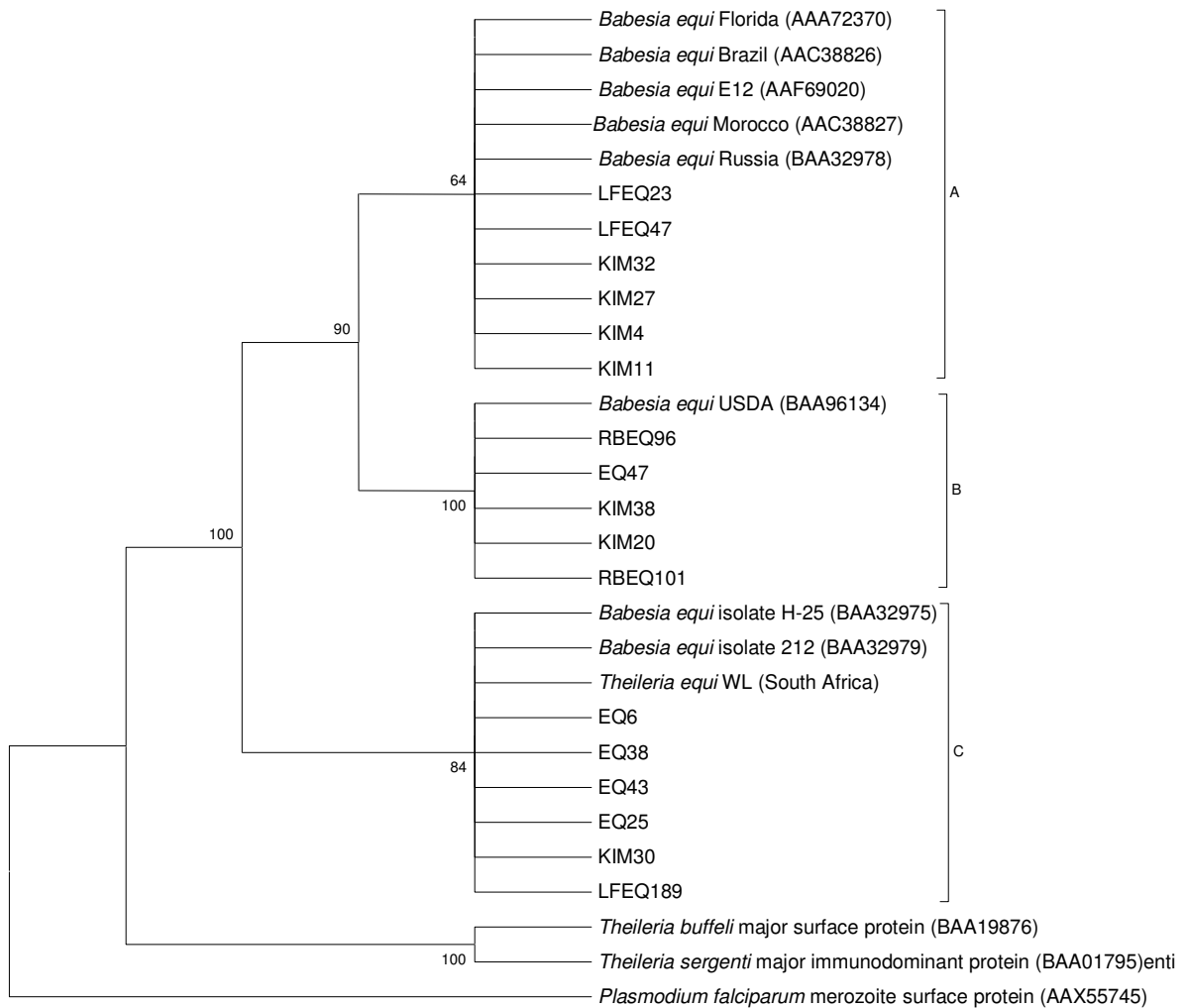
Figure 5.5 Rooted 50% majority-rule consensus cladogram, calculated using maximum-parsimony and bootstrap estimates from 1000 replicates, of the *T. equi* 18S rRNA gene sequences identified in this study and the *T. equi* 18S rRNA sequences from the public sequence databases (accession numbers are indicated in parenthesis). The *Hepatozoon canis* 18S rRNA gene sequence (accession number: DQ439543) was used as an outgroup. Bootstrap support values are indicated on the nodes of the trees.

Figure 5.6 Rooted 50% majority-rule consensus cladogram, calculated using maximum-parsimony and bootstrap estimates from 1000 replicates of representative EMA-1 amino acid sequences identified in this study and the *T. equi* EMA-1 amino acid sequences from the public sequence database (accession numbers are indicated in parenthesis). Bootstrap values are indicated on the nodes of the tree.

5.5   Discussion

The gene encoding an immunodominant surface protein of *T. equi*, equi merozoite antigen-1 (EMA-1) has previously been used as a target in the development of a TaqMan qPCR assay to determine the expression levels of merozoite surface proteins at critical stages within the tick vectors (Ueti et al., 2003). It was subsequently used to determine the ability of tick vectors to acquire and transmit *T. equi* following feeding on a chronically infected horse with low piroplasm parasitaemia (Ueti et al., 2003; Ueti et al., 2005). Recently, a modified multiplex qPCR assay combining the Ueti *ema-1* qPCR assay and a *B. caballi rap-1* TaqMan qPCR assay

was used in the detection of equine piroplasmosis in Brazil (Ueti et al., 2003; Heim et al., 2007). In all instances, the Ueti *ema-1* qPCR assay was shown to reliably amplify the *ema-1* gene and therefore detect *T. equi* infections. In this study, however, the Ueti *ema-1* qPCR assay failed to detect parasite DNA in 20 South African samples that tested positive for *T. equi* using the IFAT, cELISA, *in vitro* culture and the Kim 18S qPCR assay. Although the Ueti *ema-1* qPCR assay has previously been reported to detect *T. equi* in chronically infected horses with parasitaemias ranging from a low of 1.99 to a high of 1000 parasites/µl (Ueti et al., 2005), this assay could only detect *T. equi* in the *in vitro*-cultured South African *T. equi* WL isolate (1.38% PE) at a high C_q value. The lack of specificity of the Ueti *ema-1* qPCR primers and/or probe sequences may explain the discrepancies observed between the high Ueti *ema-1* qPCR C_q values and the lower C_q values obtained using the Kim 18S qPCR assay, in samples that were positive by both assays. These results therefore suggested that the Ueti *ema-1* qPCR assay should be reassessed and the *ema-1* gene sequences of South African *T. equi* samples determined, to ensure that genetic variation within the qPCR primer and probe sequences was not overlooked.

There are several possible explanations for the conflicting results observed for the 13 samples that tested positive by at least one of the five assays used (Table 5.2). Sero-positive but qPCR-negative results could be explained by the fact that antibodies may be present in the absence of parasites (Holman et al., 1993). Such results might also be explained by the presence of PCR inhibitors or could indicate sequence heterogeneity in the qPCR primer and/or probe regions that prevent the detection of parasite DNA. Sero-negative but qPCR-positive results could indicate a new infection prior to the development of antibodies (Donnelly et al., 1982; Bhoora et al., 2009b, Chapter 3). One of the limitations of IFAT is that it is often difficult to differentiate between weak positive and negative reactions (Weiland, 1986). The cELISA has however been shown to have higher specificities for *T. equi* when compared to IFAT (Knowles et al., 1991b; Shkap et al., 1998; Xuan et al., 2002), which would explain the IFAT-negative but cELISA-positive results. In contrast, cELISA-negative but IFAT-positive results could indicate either the genetic inability of these horses to produce antibodies to the epitope defined by the monoclonal antibody, the absence of the epitope on the *T. equi* isolates that infected these horses or the reduced sensitivity of the cELISA assay (Knowles et al., 1991b).

Sequence analysis of the *T. equi ema-1* gene from South African field samples and isolates confirmed the existence of distinct *ema-1* sequences, which belonged to three main phylogenetic groups. To ensure the stability of the *ema-1* gene sequence used as a target in the qPCR assay, Ueti et al. (2005) reported on the cloning and sequencing of the full-length (816 bp) *ema-1* gene from the Florida strain of *T. equi* (Accession number: L13784) from 11 time points over 39 months. In their study, five clones containing single nucleotide substitutions in the qPCR forward primer and probe binding regions were identified and compared to clones without any changes. Despite the presence of these single nucleotide changes, the efficiency of the qPCR assay remained unaffected (Ueti et al., 2005). Our data suggest a marked decrease in the efficiency of amplification and detection of South African *T. equi* parasites with five or more sequence differences in the primer and probe regions. An alignment of the *ema-1* fragment sequenced from 38 South African *T. equi* samples indicated that the qPCR primers and probe sequence regions were conserved among all samples occurring in group A. However, nucleotide differences in sequences occurring in groups B and C probably explain either the failure, or the decreased sensitivity of the Ueti *ema-1* qPCR assay in detecting *T. equi* in some samples.

Based on the observed variation in the South African *ema-1* gene sequences, a TaqMan MGB™ qPCR (Bhoora *ema-1* qPCR assay), targeting a more conserved region of the gene was developed for the quantitative detection of *T. equi* from South African equine blood samples. The Bhoora *ema-1* qPCR assay was highly sensitive, able to detect as low as 1.4×10^{-4}% PE. The Ueti *ema-1* qPCR assay (Ueti et al., 2003; Ueti et al., 2005) could detect *T. equi* in chronically infected horses with parasitaemias ranging from a low of 1.99 parasites/µl to a high of 1000 parasites/µl. Kim et al. (2008) determined the detection limit of their 18S rRNA-based qPCR assay (Kim 18S qPCR assay) to be 1×10^{-5}% PE; in our hands, the detection limit (95% sensitivity) of this qPCR assay was 1.9×10^{-4}% PE (Bhoora et al., 2009b; Chapter 3). The sensitivity of the Bhoora *ema-1* qPCR assay is therefore comparable to that reported for the Kim 18S qPCR assay. Furthermore, no amplification could be observed from DNA of other protozoal parasites expected to occur in equids, thus indicating the high specificity of the assay. When tested for its ability to detect *T. equi* parasite DNA from the 107 field samples collected from horses at the National Yearling sale (2006), the Bhoora *ema-1* qPCR assay was shown to be more sensitive than the Ueti *ema-1* qPCR assay.

Other molecular assays that target the *ema-1* gene of *T. equi* have also been developed (Battsetseg et al., 2001; Nicolaiewsky et al., 2001; Alhassan et al., 2005; Salim et al., 2008). Using nested PCR, Nicolaiewsky et al. (2001) reported the amplification of a 102 bp *ema-1* fragment from equine blood samples with a parasitaemia as low as 6 x 10^{-6}%. In comparison, the Bhoora *ema-1* qPCR assay could detect *T. equi* at 1.4 x 10^{-4}% PE. However, real-time PCR technology provides several advantages over the use of conventional and nested PCR assays: there is no need for post-PCR manipulation which reduces the risk of contamination, and it is possible to quantify the amount of target DNA in the reaction. Although the internal primers (EMAI-F and EMAI-R), designed by Nicolaiewsky et al. (2001) to amplify the 102 bp fragment, would have been able to amplify all sequences occurring in group A, two and three nucleotide differences were observed in the EMAI-F primer region of sequences occurring in groups B and C, respectively. No nucleotide variations were observed in the EMAI-R primer region for sequences occurring in these two groups. These differences, although minor, may result in a reduced efficiency of amplification.

The *T. equi* qPCR assays were evaluated by testing their ability to detect parasites in 41 South African field samples (Table 5.4). Significant correlation (90%) was observed between the IFAT and the Bhoora *ema-1* qPCR assay results. The Ueti *ema-1* qPCR assay detected *T. equi* in fewer samples (80%) than the Bhoora *ema-1* qPCR assay; the results could be attributed to the observed variation in the *ema-1* gene, as discussed earlier. Both the Kim 18S and the Ueti *ema-1* qPCR assays failed to detect *T. equi* parasite DNA in four samples (Kim11, Kim20, Kim31 and Kim40) that could be detected using the Bhoora *ema-1* qPCR assay. Of these, we were only able to amplify and sequence the *ema-1* gene from Kim20. Phylogenetic analysis indicated that this sample grouped in clade B (Figure 5.6) and sequences occurring in this clade contained between one and seven nucleotide differences in the regions where the Ueti *ema-1* qPCR primers and probe had been designed. The *ema-1* sequence from Kim20 had three nucleotide differences in the forward primer region and one difference each in the reverse primer and probe regions (results not shown). The qPCR primers and probes developed by Kim et al. (2008) were designed in a region close to the 5'-end of the 18S rRNA gene. Again, we were only able to amplify and sequence the 5' fragment of the 18S gene from Kim20. Inspection of the 18S rRNA sequence of Kim20 indicated the occurrence of a single nucleotide difference in the qPCR forward primer region, while no differences were observed in the reverse primer and probe regions (results not shown). Single nucleotide variations in the qPCR forward primer region, previously reported for twelve South African *T. equi* variants, did not affect the ability of the

Kim 18S qPCR assay to detect each of these variants (Bhoora et al, 2009b; Chapter 3) and single nucleotide changes did not affect the efficiency of the Ueti *ema-1* qPCR assay (Ueti et al., 2005). Since we could not amplify and sequence the 5' end of the 18S rRNA gene from the other three samples, we can only speculate that single nucleotide changes in combination with low piroplasm parasitaemias (as indicated by high C_q values obtained using the Bhoora *ema-1* qPCR assay) may compromise the efficiencies of the qPCR assays, thus yielding negative results.

Recently, a multiplex qPCR assay targeting genes encoding antigenic proteins of *T. equi* and *B. caballi*, has been developed for the detection of equine piroplasmosis (Heim et al., 2007). The multiplex qPCR assay (MRT-PCR), described by Heim et al. (2007), was developed using the qPCR primers and probes initially described by Ueti et al. (2003). Based on the data presented in this study, it is evident that the MRT-PCR assay will not be able to detect all *T. equi ema-1* genotypes identified in South Africa. Redesigning of the MRT-PCR assay using the newly developed TaqMan MGB™ assay described in this study may overcome this problem.

Three distinct clades were identified by phylogenetic analysis of both *T. equi* 18S rRNA gene sequences and EMA-1 amino acid sequences. A comparison between the *T. equi* 18S rRNA and *T. equi* EMA-1 phylogenetic trees showed that samples which grouped in one *T. equi* 18S genotype did not group together in a particular EMA-1 phylogenetic clade, but rather occurred randomly between the three EMA-1 clades identified.

Ribosomal RNA (rRNA) genes are thought to be under tight structural and functional constraint, resulting in lower substitution rates; this and the absence of lateral gene transfer across lineages, are characteristics which make rRNA genes appropriate targets to assist in species identification (Chae et al., 1998; Katzer et al., 1998; Allsopp and Allsopp, 2006). Our previous studies have shown that extensive sequence heterogeneity exists in the 18S rRNA gene of *T. equi* parasites of both horses and zebra in South Africa (Bhoora et al., 2009a; Chapter 2; Chapter 4). It is probably not possible to use the 18S rRNA gene alone to decide whether these variants represent new species or subspecies (Chae et al., 1999; Allsopp and Allsopp, 2006). On the other hand, parasite outer membrane protein gene sequences, which are likely to be under intense selection pressure and therefore evolve at a more rapid rate than core function genes, will probably not give reliable phylogenetic information at the species level (Allsopp and Allsopp, 2006). In a study conducted to establish the phylogenetic relationship between different *Theileria* species, a comparison between homologous major merozoite/piroplasm surface antigen genes (mMPSA)

and the ribosomal rRNA genes from the different *Theileria* species investigated, showed that even though the phylogenetic clades were consistent with respect to the major groupings, inconsistencies between the analysis with the two types of gene sequence were found within the groups (Katzer et al., 1998). In addition, Katzer et al. (1998) identified four *Bems* gene sequences (*Bems*1-1, *Bems*1-2, *Bems*1-3 and *Bems*1-4) derived from genomic DNA of the *T. equi* (Florida) stock, which showed between 93.6-65.5% nucleotide identity to each other. The *Bems*1-3 sequence was found to be closely related to the *ema-2* sequence identified from the Florida isolate of *T. equi* (Knowles et al., 1997; Katzer et al., 1998). The recent identification and characterization of the *T. equi* EMA-3 suggests that these homologues either represent members of a related gene family within the genome of *T. equi* or distinct variants of the same gene (Katzer et al., 1998; Kumar et al., 2004; Ikadai et al., 2006). Such differences are not surprising, as surface exposed mMPSA genes are expected to be under selection pressure and have therefore evolved molecular mechanisms that generate diversity as a means of evading the immune system and succeeding in parasitism (Carcy et al., 2006). The use of these divergent mMPSA gene sequences may therefore be preferable for the comparison of closely related species (Shiels et al., 1995; Katzer et al., 1998).

The occurrence of such sequence variation may be related to antigenic variability and pathogenicity (Katzer et al., 1998). This has been demonstrated in a recent study on the association of *B. rossi* genotypes and the clinical manifestation of canine babesiosis (Matjila et al., 2009). Phylogenetic analyses of *Babesia rossi* erythrocyte membrane antigen 1 (Br*EMA1*) gene sequences enabled the identification of 13 Br*EMA1* genotypes, four of which could be linked to mild, moderate or severe disease phenotypes (Matjila et al., 2009). Unfortunately, it is not known whether the different *T. equi* 18S rRNA or *ema-1* genotypes can be associated with clinical differences in equine piroplasmosis cases.

In conclusion, this study reports on the development and evaluation of a sensitive TaqMan MGB™ qPCR assay, targeting the *ema-1* gene for the detection of *T. equi* infection in horses. Taking into consideration the discovery of extensive sequence heterogeneity both within the *ema-1* gene and the *T. equi* 18S rRNA gene, the use of this highly sensitive and specific *ema-1* qPCR assay, in conjunction with the *T. equi* 18S qPCR assay (Kim et al., 2008; Bhoora et al., 2009b; Chapter 3), may assist in the improved detection and diagnosis of equine piroplasmosis.

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CHAPTER 6

Sequence heterogeneity in the gene encoding the

rhoptry associated protein-1 (RAP-1) of

Babesia caballi isolates from South Africa

6.1   Abstract

A competitive-inhibition enzyme-linked immunosorbent assay (cELISA) developed for the detection of antibody specific for *Babesia caballi* was used to test sera collected from 1237 South African horses. None of these samples tested positive using the cELISA, although 63 samples tested positive for *B. caballi* antibody using the indirect fluorescent antibody test (IFAT). We therefore characterized the *rap-1* gene that codes for the antigen (rhoptry associated protein, RAP-1) used in the cELISA, from South African *B. caballi* isolates. Three sets of primers were designed to amplify the complete gene and flanking regions (~1800 bp), but only one set of primers yielded PCR products, and we were only able to amplify a region at the 5' end of the gene (615 bp) from ten South African *B. caballi in vitro*-cultured isolates. Sequence data from seven of these were obtained. The sequences showed between 79 and 81% identity to *B. caballi rap-1* gene sequences that have been reported in the literature (accession numbers: AF092736 and AB017700). The GenomeWalker Universal kit (Clonetech) was used to amplify the regions flanking the 615 bp *B. caballi rap-1* fragment from two South African isolates. Amplified products were cloned into the pGEM-T Easy vector and sequenced. The complete *rap-1* gene sequence, comprising a single open reading frame of 1479 bp that encodes a protein consisting of 493 amino acids, was obtained from the two South African isolates. These sequence data were used to redesign the amplification primers and *rap-1* homologues were obtained from a further eight isolates. BLASTP analysis indicated an amino acid identity of between 57.9% and 65.1% to the two RAP-1 protein sequences, AF092736 and AB017700, with most differences occurring at the carboxy-terminus. The amino acid sequence differences probably explain why it was not possible to detect *B. caballi* antibody in IFAT-positive sera from South Africa using the cELISA. Redesigning the current cELISA using a conserved epitope of the RAP-1 antigen, or a more conserved protein as the target antigen, may overcome this problem.

6.2   Introduction

Babesia caballi is a tick-borne haemoprotozoan parasite, and is one of the causes of equine piroplasmosis. The clinical manifestations of the disease are often variable, making it easy to confuse with *Theileria equi* infections. *Babesia caballi* infections, characterized by fever and anaemia, are considered less severe than *T. equi* infections, which are more commonly associated with haemoglobinuria and death (de Waal, 1992; Camacho et al., 2005). Many

infections caused by either parasite are subclinical and, in addition, animals that have recovered from the infections often remain carriers of the parasites for long periods of time.

The international movement of horses has led to the spread of equine piroplasmosis from its endemic tropical and subtropical regions to more temperate non-endemic regions. Many countries have introduced stringent import restrictions to prevent the introduction of these parasites into disease-free areas (Friedhoff et al., 1990). Serological methods of determining the carrier status of horses and other equid species are currently the prescribed methods for certifying animals free of these parasites. These include the indirect fluorescent antibody test (IFAT) and enzyme-linked immunosorbent assay (ELISA) (Donnelly et al., 1980; Weiland, 1986; Brüning et al., 1997). Although it is possible to distinguish between *T. equi* and *B. caballi* infections using IFAT, differentiation between weak positive and negative reactions can be difficult. Cross-reactions that occur between *B. caballi* and *T. equi* in the indirect ELISA prevent this test from being recognized as a differential diagnostic assay (Weiland, 1986). Recently, however, *T. equi* and *B. caballi* recombinant merozoite proteins and monoclonal antibodies to immunogenic epitopes on these proteins have been used in competitive inhibition ELISAs (cELISAs), with promising results. These cELISAs have been shown to have higher specificities for *T. equi* and *B. caballi*, when compared to those of the IFAT and indirect ELISA (Knowles et al., 1991; Knowles et al., 1992; Shkap et al., 1998; Kappmeyer et al., 1999; Katz et al., 2000; Xuan et al., 2002).

Secreted proteins from the apical organelles of apicomplexan parasites are thought to play pivotal roles in parasite attachment to, invasion of and expansion and maintenance within the host cell (Sam-Yellowe, 1996). Among these proteins is the rhoptry-associated protein-1 (RAP-1), which was initially described in *Babesia bovis* and *Babesia bigemina*, but has subsequently been described in other *Babesia* parasites (Dalrymple et al., 1993; Skuce et al., 1996; Suarez et al., 1998; Kappmeyer et al., 1999; Ikadai et al., 1999). The RAP-1 family of proteins contains several immunogenic epitopes and antibodies directed against these proteins have been shown to inhibit merozoite invasion (Ikadai et al., 1999; Machado et al., 1999; Yokoyama et al., 2006). This phenomenon suggests that RAP-1 proteins are important targets of the protective immune response (Suarez et al., 2003).

A monoclonal antibody to recombinant RAP-1 was used in the development of a cELISA for the detection of *B. caballi* antibody in infected horses (Kappmeyer et al., 1999). This assay has been successfully used for the detection of *B. caballi* antibody in the sera of infected horses in North and South America and several European countries (Kappmeyer et al., 1999; Sevinc et al., 2008). A preliminary study in our laboratory, however, showed that the commercially available cELISA was not able to detect antibody in South African horses infected with *B. caballi*. This result led to the hypothesis that differences in the RAP-1 antigen within South African *B. caballi* isolates could prevent the detection of *B. caballi* antibody. This study was therefore focused on the characterization of *rap-1* gene homologues in South African *B. caballi* isolates in an attempt determine the cause of the failure of the commercial cELISA in South Africa.

6.3 Materials and Methods

6.3.1 Field samples and in vitro-cultured isolates

A total of 1237 whole blood samples were collected from horses at the National Sale of two-year-old thoroughbred horses in 2005 (n=273) and the National Yearling Sales in 2005 (n=455) and 2006 (n=509). Ten *in vitro*-cultured *B. caballi* isolates, designated Bcab5, Bcab9, Bcab13, Bcab19, Bcab105, Bcab167, Bcab418, Bcab443, Bcab502 and BcabE7 (Zweygarth et al., 2002), were also used in this study.

6.3.2 cELISA and IFAT

Sera obtained from the blood samples were examined for the presence of antibodies against *B. caballi* and *T. equi* using the commercially available cELISA kit, as described by the manufacturer (VMRD Inc., Pullman, WA, USA). The cELISA was performed at the Department of Veterinary Tropical Diseases.

The IFAT was conducted at the Agricultural Research Council-Onderstepoort Veterinary Institute (ARC-OVI). A standard IFAT protocol, as described in the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (Anonymous, 2008) was used, with the exception that cultured antigen, produced locally in South Africa, was used.

6.3.3 DNA extraction, PCR amplification and sequencing

Genomic DNA was extracted from 200 µl of each of the *in vitro*-cultured isolates using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Primers were designed to amplify complete *rap-1* homologues and flanking sequences (~1800 bp) from the South African isolates based on two *B. caballi rap-1* DNA sequences available in the public sequence databases [accession numbers: AF092736 (Kappmeyer et al., 1999) and AB017700 (Ikadai et al., 1999)]. Initially, primers BC-RAP1F and BC-RAP1R (Table 6.1, Figure 6.1) were designed to amplify the full-length gene and flanking sequences. Subsequently, primer pairs BC-RAP1F and BC-RAP3R, BC-RAP2F and BC-RAP2R, and BC-RAP3F and BC-RAP1R (Table 6.1) were designed to amplify overlapping fragments, respectively, at the 5' end, in the middle and at the 3' end of the ~1800 bp *rap-1* fragment (Figure 6.1). The FastPCR software program (http://www.biocenter.helsinki.fi/bi/Programs/fastpcr.htm) was used to design all primers used in this study, and to check for the formation of secondary structures and primer dimers. All PCR primers were obtained from Integrated DNA Technologies (IDT). Reactions were performed in a final volume of 25 µl, containing High Fidelity PCR Master mix (Roche Diagnostics, Mannheim, Germany), 0.2 µM of each primer and 30 ng of genomic DNA. The cycling conditions were: an initial denaturation of 5 min at 95 ºC, followed by 40 cycles of 30 s at 95 ºC, 1 min at 55 ºC and 1 min at 72 ºC, and a final extension of 7 min at 72 ºC.

PCR products were purified using the Qiagen PCR purification kit (Qiagen, Hilden, Germany). Samples were sequenced using BigDye chemistry (v.3.1, Applied Biosystems) on a 3130XL genetic analyser (Applied Biosystems). Sequences were assembled and edited using gap4 of the Staden software suite (Staden, 1996). Multiple sequence alignments were performed using the MAFFT alignment program (Multiple Sequence Alignment) employing the FFT-NS-1 algorithm (Katoh et al., 2005). The alignments were adjusted manually using BioEdit version 7.0.5.2 (Hall, 1999). Searches of databases for homologous nucleotide sequences were performed using BLASTN and for homologous protein sequences, BLASTP was used (Altschul et al., 1990).

Table 6.1   Nucleotide sequences of PCR primers used in this study.

| Primer	Size (bp)	Sequence (5'- 3')	Tm	%GC
BC-RAP1F	23	CGTACAATGAGGTGTTCTGCGAG	57.8	52.1
BC-RAP1R	22	TATTCTCCGAGCAGTGCGATGG	59.0	54.5
BC-RAP2F	21	AGAGTGACGCTAATCCGGCCA	60.7	57.1
BC-RAP2R	23	GAATAAATCCTCGGCCCCCGTTG	60.2	56.5
BC-RAP3F	21	AGTGTCAACGGGGGCCGAGGA	65.5	66.6
BC-RAP3R	23	CGTCTTGTAGTAAAGCGTGGCCA	59.4	52.1
BC-GSP1F	30	CTGGATGCGTTTCAGGAGTGGCAAGAACCA	65.3	53.3
BC-GSP1R	28	CGCTACACGGCTGTTGTGGCGAATGGCC	68.5	64.2
BC-GSP2F	30	GGAGAAGAACGTGACTAGCGACCCCAACGT	65.8	56.6
BC-GSP2R	30	CCTTGCTACAAGCAAGAGGGCGCCGAAAAC	66.2	56.6
Bc9_RAPF	24	AGCAGTGCTGTATATGTCTGTGTC	56.7	45.8
Bc9_RAPR	23	GCTGATGCGATGTGTGTCGTAGG	60.0	56.5
Bc9_RAP2F	24	ACTAGCGACCCCAACGCTACTGAC	62.4	58.3
Bc9_RAP2R	22	TTGGAGCATGAAGTCCTTCAGC	57.5	50.0
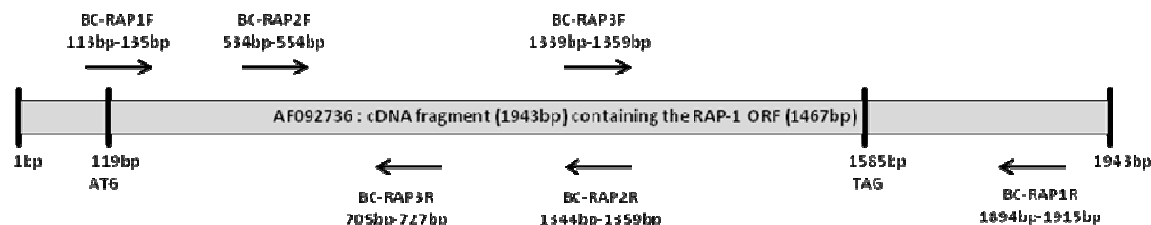
Figure 6.1   Schematic representation of positions of primers for amplification of *rap-1* gene homologues and flanking regions from South African *B. caballi* isolates.

6.3.4   *Genome Walking*

Since only one fragment close to the 5' end of the *rap-1* gene could be obtained by PCR amplification, the GenomeWalker™ Universal Kit (CloneTech) was used to amplify and sequence the remaining 5' and 3' flanking regions of two South African *B. caballi in vitro-*cultured isolates (Bcab9 and Bcab13).   Genomic DNA from *B. caballi* isolates Bcab9 and Bcab13 were digested with four blunt-end restriction enzymes, *Dra*I, *Eco*RV, *Pvu*II and *Stu*I. GenomeWalker adaptors (Table 6.2) were ligated to 4 µl of each digested genomic DNA library.

Table 6.2   Nucleotide sequences of the GenomeWalker Adaptor and adaptor primers.

Oligonucleotide	Nucleotide Sequence
Adaptor	5'-GTAATACGACTCACTATAGGGCACGCGTGGTCGACGGCCCGGGCTGGT-3'
	3' H_2N-CCCGACCA-PO_4-5'
Primer ADP1	5'-GTAATACGACTCACTATAGGGC-3'
Primer ADP2	5'-ACTATAGGGCACGCGTGGT-3'

Four gene-specific primers, BC-GSP1F, BC-GSP1R, BC-GSP2F and BC-GSP2R (Table 6.1), were designed for the amplification of the 5' and the 3' unknown flanking regions of the *rap-1* gene. Primary PCR reactions were performed using adaptor-specific primer ADP1 (Table 6.2) and gene-specific primer BC-GSP1R for the amplification of the 5' end of the *rap-1* gene, while ADP1 and BC-GSP1F were used to amplify the 3' end. Secondary PCR reactions were carried out using the adaptor-specific primer ADP2 (Table 6.2) and gene-specific primer BC-GSP2R to amplify the 5' end, and ADP2 and BC-GSP2F to amplify the 3' end of the *rap-1* gene. Both primary and secondary PCR reactions were carried out according to the manufacturer's instructions.

The resulting secondary PCR products were purified using a QIAquick gel extraction kit (Qiagen, Hilden, Germany) and cloned using the pGEM®-T Easy Vector system II (Promega), according to the manufacturer's instructions. Transformants were screened using ImMedia™ Amp Blue (Invitrogen), and recombinant colonies were selected and grown using ImMedia™ Amp Liquid (Invitrogen). Plasmid DNA was isolated by means of a High Pure Plasmid Purification kit (Roche) and sequenced using the cloning vector primers, T7 and SP6. Sequences were edited and analysed as described above.

6.3.5   *Amplification and sequencing of the rap-1 gene homologue from South African B. caballi isolates*

An approximately 1500 bp fragment containing the *rap-1* gene was amplified from another eight South African *B. caballi in vitro*-cultured isolates using the primers, Bc9_RAPF and Bc9_RAPR (Table 6.1), that were designed based on the *rap-1* sequence data obtained from the South African *B. caballi* isolates Bcab9 and Bcab13. Reactions were performed in a final volume of 25 μl, containing High Fidelity PCR Master mix (Roche Diagnostics, Mannheim, Germany), 0.2 μM of each primer and 30 ng of genomic DNA. The cycle parameters included an initial denaturation of 5 min at 94 ºC, followed by 40 cycles of 30 s at 94 ºC, 1 min at 58 ºC and 1 min

at 72 ℃, and a final extension of 7 min at 72 ℃. Amplicons were purified and sequenced as described above using the amplification primers, Bc9_RAPF and Bc9_RAPR, and two internal sequencing primers, Bc9_RAP2F and Bc9_RAP2R (Table 6.1). The sequences were aligned and edited as described above. Representative South African *B. caballi rap-1* gene sequences have been deposited in GenBank under accession numbers GQ871778, GQ871779 and GQ871780.

6.3.6   Phylogenetic analysis

Rap-1 nucleotide sequences were aligned using the MAFFT alignment program (Multiple Sequence Alignment) employing the FFT-NS-1 algorithm (Katoh et al., 2005), then translated into amino acid sequences using the ExPasy translate tool (www.expasy.ch/tools/dna). Modeltest v 3.7 (Posada and Crandall, 1998) was used to select a TrN + I + G substitution model with a proportion of invariable sites and a gamma-shaped distribution of rates across sites. PAUP* v4b10 (Swofford, 2003) was used to explore distance, parsimony and maximum-likelihood phylogenetic methods for both nucleotide and amino acid sequences. Trees were constructed using the *Babesia divergens rap-1* gene sequence (Accession number: Z49818) as an outgroup. Analysis by Bayesian inference was performed using MrBayes v3.1.2 (Ronquist and Huelsenbeck, 2003) which is accessible via the Computational Biology Service Unit at Cornell University (http://cbsuapps.tc.cornell.edu/mrbayes.aspx). All consensus trees were edited using MEGA4 (Tamura et al., 2007).

6.3.7   Analysis of the 18S rRNA gene from South African in vitro-cultured B. caballi isolates

The complete 18S rRNA gene of the *in vitro*-cultured *B. caballi* isolates was amplified and sequenced as described previously (Bhoora et al., 2009; Chapter 2), and phylogenetic analyses using distance, parsimony and maximum-likelihood methods were carried out using the program PAUP* v4b10 (Swofford, 2003). Trees were constructed using the 18S rRNA gene sequence of *B. divergens* (accession number: Z48751) as an outgroup.

6.4 Results

Of the field samples (n=1237) tested for the presence of *B. caballi* and *T. equi* antibodies using the commercially available cELISA kits, none of the samples tested positive for *B. caballi* antibodies, but 265 samples were positive for *T. equi* antibodies. The samples were also tested using the IFAT, which detected *B. caballi* antibodies in 63 samples and *T. equi* antibodies in 274 samples (Table 6.3).

No amplification product could be obtained from any of the ten South African *B. caballi in vitro-*cultured isolates using the BC-RAP1F and BC-RAP1R primer pair. Of the primers designed to amplify overlapping fragments at the 5' end, in the middle and at the 3' end of the *rap-1* fragment (Figure 6.1), only one primer pair, BC-RAP1F and BC-RAP3R, yielded a ~615 bp PCR product from the ten South African *B. caballi* isolates, and sequence data were obtained from seven of these. BLASTN analysis revealed that the sequences showed between 79 and 81% identity to published *B. caballi rap-1* nucleotide sequences (Table 6.4). Given this sequence heterogeneity at the 5' end of the *rap-1* gene, we hypothesized that even greater sequence heterogeneity present in the middle and at the 3' end of the *rap-1* gene in South African isolates prevented the downstream primers from binding to the template DNA, resulting in no amplification. An alternative method was therefore required to obtain full-length *rap-1* gene sequences from the South African *B. caballi* isolates.

Table 6.3   Comparison of serology results for (A) *B. caballi* and (B) *T. equi*.

(A) *B. caballi*

	cELISA positive	cELISA negative	Total
IFAT positive	0	63	**63**
IFAT negative	0	1174	**1174**
Total	**0**	**1237**	**1237**

(B) *T. equi*

	cELISA positive	cELISA negative	Total
IFAT positive	265	9	**274**
IFAT negative	0	963	**963**
Total	**265**	**972**	**1237**

Table 6.4 PCR and sequencing results obtained for *rap-1* homologues from ten South African *B. caballi in vitro*-cultured isolates.

Isolate	*rap*-1 PCR*	Sequencing result**
Bcab5	+	568 bp; 80% identity
Bcab9	+	591 bp; 80% identity
Bcab13	+	590 bp; 80% identity
Bcab19	+	417 bp; 77% identity
Bcab105	+	514 bp; 78% identity
Bcab341	+	573 bp; 80% identity
Bcab418	+	No sequence data
Bcab443	Multiple bands	No sequence data
Bcab502	+	518 bp; 79% identity
BcabE7	Double bands	No sequence data

* Amplification products obtained using primers BC-RAP1F and BC-RAP3R

**Number of base pairs sequenced and % identity to published RAP-1 sequence AF092736

Two *B. caballi in vitro*-cultured isolates (Bcab9 and Bcab13) were selected for subsequent genome walking experiments. The primary PCR using adaptor primer 1 (ADP1) and gene-specific primers (BC-GSP1F or BC-GSP1R) produced either smearing in some lanes or multiple fragments that ranged in size from 500 bp to 3000 bp. The secondary PCR using adaptor primer 2 (ADP2) and gene-specific primers (BC-GSP2F or BC-GSP2R) produced distinct PCR products in each of the four libraries for the 3' unknown region (Figure 6.2). For amplification of the 5' unknown region, PCR products were observed in all libraries except the *Stu*I digested library (Figure 6.2).
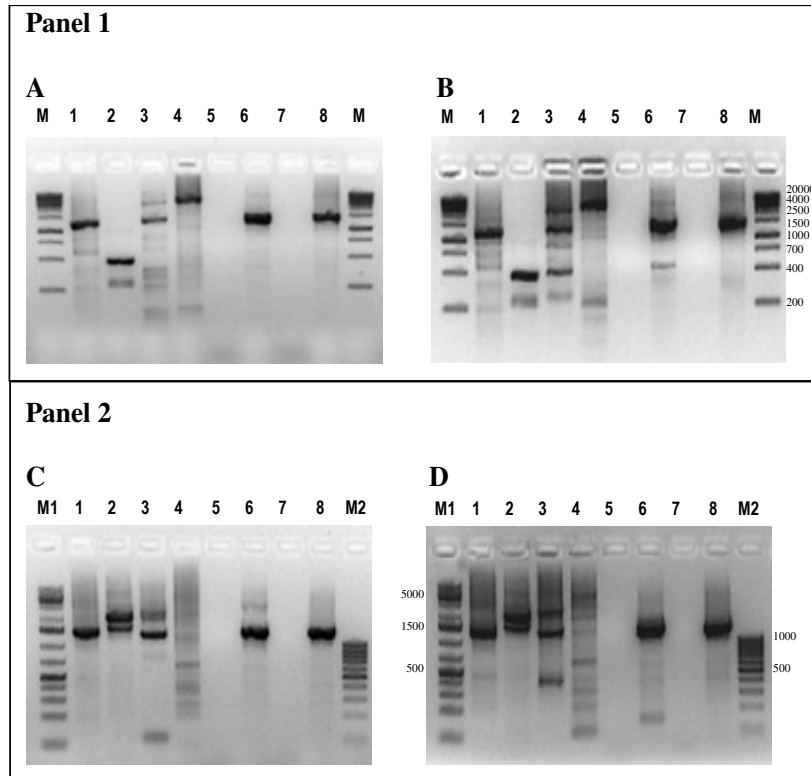
Figure 6.2   Genome walking PCR analysis of restriction enzyme digested genomic DNA using primers ADP2 and BC-GSP2F to amplify the 3'-end of the *rap-1* fragment (Panel 1) and primers ADP2 and BC-GSP2R to amplify the 5'-end (Panel 2).   (A and C) *B. caballi in vitro*-cultured isolate Bcab9. (B and D) *B. caballi in vitro*-cultured isolate Bcab13.   Lane 1: *Pvu*II library. Lane 2: *Dra*I library. Lane 3: *Eco*RV library. Lane 4: *Stu*I library. Lanes 5 and 7: negative controls. Lanes 6 and 8: Positive control (pre-constructed human genomic DNA library). Lane M: ZipRuler Express DNA ladder 2 (Fermentas). Lane M1: O'GeneRuler 1 kb DNA ladder plus DNA size markers (Fermentas). Lane M2: O'GeneRuler 100 bp DNA ladder (Fermentas).

PCR and sequence data generated from the *Pvu*II digested genome walking libraries were used in the construction of the complete *B. caballi* isolate 9 and *B. caballi* isolate 13 *rap-1* sequences. A single open reading frame of 1479 bp, encoding a peptide of 493 amino acids, was obtained from the two South African *B. caballi* isolates. BLAST analysis of the sequences confirmed that they were most closely related to the *B. caballi* RAP-1 family. The two South African *B. caballi* *rap-1* sequences were very similar to each other, with 99% nucleotide sequence identity, but they showed much lower identity (81.7 and 82.3%) to two published *B. caballi rap-1* sequences, AF092736 (Kappmeyer et al., 1999) and AB017700 (Ikadai et al., 1999) (Table 6.5).

Table 6.5 Nucleotide sequence identity of *rap*-1 sequences from the South African *B. caballi* isolates compared to published sequences AB017700 (Ikadai et al., 1999) and AF092736 (Kappmeyer et al., 1999) as determined by BLASTN analysis.

	AB017700 % identity	AF092736 % identity	Bcab9 % identity
AB017700	100.0	99.9	81.8
AF092736	99.9	100.0	82.3
Bcab5	81.9	82.4	99.4
Bcab9	81.8	82.3	100.0
Bcab13	81.7	82.1	99.3
Bcab19	82.0	82.0	99.3
Bcab105	81.9	82.4	99.4
Bcab167	81.9	82.0	87.5
Bcab418	82.4	82.4	89.8
Bcab443	81.9	82.0	87.6
Bcab502	81.8	82.3	99.4
BcabE7	82.0	82.0	87.5

Table 6.6 Amino acid sequence identity and similarity of predicted RAP-1 amino acid sequences from the South African isolates compared to published sequences AB017700 (Ikadai et al., 1999) and AF092736 (Kappmeyer et al., 1999) as determined by BLASTP analysis

	AB017700		AF092736		Bcab 9	
	% identity	% similarity	% identity	% similarity	% identity	% similarity
AB017700	100.0	100.0	99.8	99.8	63.7	77.5
AF092736	99.8	99.8	100.0	100.0	64.9	78.6
Bcab5	63.7	77.9	64.9	78.9	98.3	98.9
Bcab9	63.7	77.5	65.1	78.6	100.0	100.0
Bcab13	63.2	77.3	64.7	78.5	98.6	98.9
Bcab19	63.7	77.5	63.7	77.5	98.2	98.7
Bcab105	59.8	73.6	61.1	74.7	93.6	94.2
Bcab167	57.9	73.2	59.2	74.2	74.2	80.7
Bcab418	62.0	76.0	62.0	76.0	76.5	82.1
Bcab443	57.9	73.2	59.5	74.3	74.4	80.9
Bcab502	63.7	77.9	63.8	78.1	98.9	80.9
BcabE7	57.9	73.2	57.9	73.0	73.2	79.7

Primers Bc9_RAPF and Bc9_RAPR (Table 6.1), which were designed based on the *rap-1* sequences obtained from *B. caballi* isolates Bcab9 and Bcab13, were used to amplify and sequence almost complete *rap-1* open reading frames (~1500 bp) from an additional eight South African *B. caballi in vitro*-cultured isolates. *Rap-1* sequences similar to those obtained from

Bcab9 and Bcab13 were obtained from isolates Bcab5, Bcab19, Bcab105, and Bcab502, but a second distinct group of *B. caballi rap-1* nucleotide sequences was identified from Bcab167, Bcab418, Bcab443 and BcabE7, with nucleotide identities to Bcab9 ranging from 87.5 to 89.8% (Table 6.5). All ten of the South African *rap-1* sequences showed between 81.7 and 82.4% identity to the two published *B. caballi rap-1* sequences (Table 6.5). BLASTP analysis, on the other hand, indicated amino acid identities to the published *B. caballi* RAP-1 protein sequences which ranged from 57.9 to 65.1%, and amino acid similarities ranging between 73.0 and 78.9% (Table 6.6).

Nucleotide sequence alignments demonstrated that sequence variation between published sequences and their homologues from South African isolates occurred across the full-length of the *rap-1* gene (results not shown). An alignment of the amino acid sequences deduced from the *rap-1* nucleotide sequences of PCR products amplified from genomic DNA, showed that the RAP-1 amino acid sequences were fairly conserved in the amino-terminal region of the protein, but were more variable at the carboxy-terminus (Figure 6.3). Distinctive features of the RAP-1 family, including the presence of a signal peptide sequence and four conserved cysteine residues (Suarez et al., 1998), could be identified (Figure 6.3). The detection of putative conserved domains by BLASTP analysis further confirmed that the newly discovered South African *B. caballi* RAP-1 sequences belong to the RAP-1 superfamily.

Figure 6.3 Alignment of the RAP-1 predicted amino acid sequences from ten South African *B. caballi* isolates (Bcab5, Bcab13, Bcab9, Bcab19, Bcab105, Bcab167, Bcab418, Bcab443, Bcab502 and BcabE7) with the published RAP-1 amino acid sequences from the public sequence databases (accession numbers: AB01770 and AF092736). The potential signal peptide cleavage site is indicated by an arrow and conserved cystein residues are highlighted in yellow. The carboxy-terminal amino acid repeat regions are indicated in blocks. Identical sequences are indicated by dots (…) and missing sequences or gaps are indicated by dashes (---).

Phylogenetic analyses of both the *rap-1* nucleotide sequences and the predicted amino acid sequences yielded trees with almost identical topologies and high bootstrap or nodal support values. The analyses demonstrated that the *B. caballi* RAP-1 amino acid sequences fell into two main groups (Figure 6.4). Group A contained the RAP-1 amino acid sequences from *B. caballi* clone X6 (AF092736) (Kappmeyer et al., 1999) and the *B. caballi* pBC48/31 mRNA for a 48 KDa merozoite antigen (AB017700) (Ikadai et al., 1999), both of which were obtained from the USDA *B. caballi* strain. Group B, which could be further subdivided into groups B1 and B2, contained the new *B. caballi* RAP-1 amino acid sequences from South African isolates. Group B1 contained RAP-1 amino acid sequences from six *B. caballi* isolates (Bcab5, Bcab9, Bcab13, Bcab19, Bcab105 and Bcab502), while the remaining four RAP-1 amino acid sequences (Bcab167, Bcab418, Bcab443 and BcabE7) were found in group B2.

Phylogenetic analyses of complete 18S rRNA gene sequences from nine of the *in vitro*-cultured samples revealed that the sequences grouped within Clade B, which has previously been shown to include only 18S rRNA sequences from South African *B. caballi* samples (Bhoora et al., 2009; Chapter 2) (Figure 6.5). The 18S rRNA sequences from six of the *B. caballi* isolates (Bcab5, Bcab9, Bcab13, Bcab19, Bcab105 and Bcab502) were shown to group together in subgroup B1 with the original South African *B. caballi* sequence (accession number: Z15104), while the remaining three isolates (Bcab167, Bcab418 and Bcab443), grouped within the second subgroup, B2, that contained the recently published South African sequence CABRBEQ115 [accession number: EU642514 (Bhoora et al., 2009; Chapter 2)] (Figure 6.5).
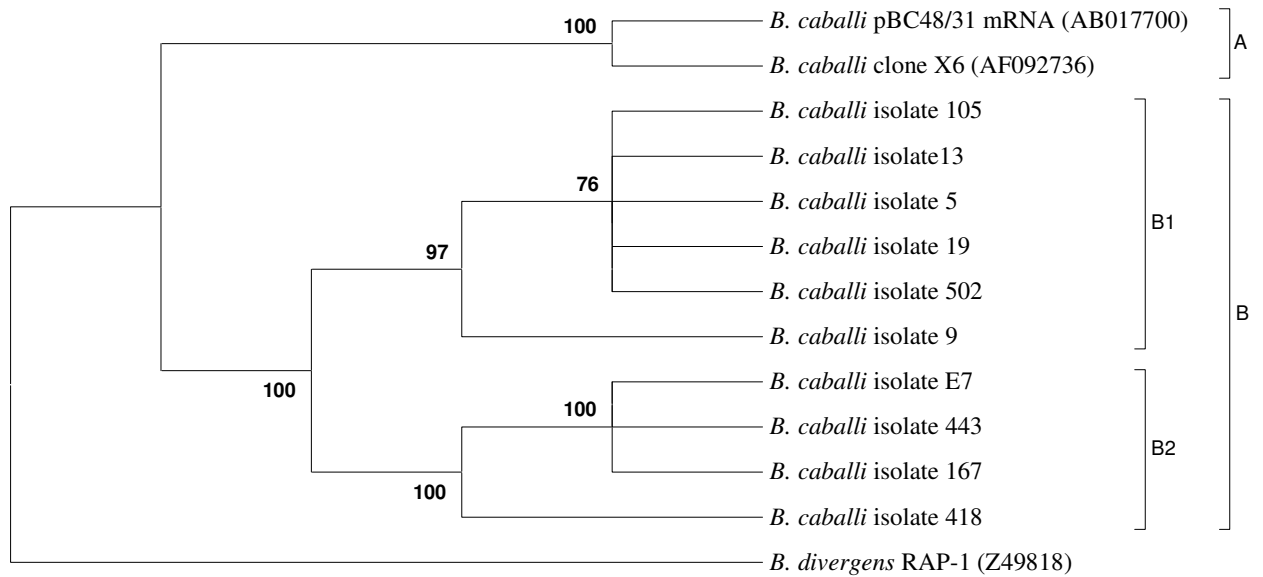
Figure 6.4   Rooted 50% majority-rule consensus cladogram, calculated using maximum-parsimony and bootstrap estimates from 1000 replicates, of the predicted *B. caballi* RAP-1 amino acid databases (accession numbers are indicated in parentheses). The *B. divergens* RAP-1 amino acid sequence (accession number: Z49818) was used as an outgroup. Bootstrap support values are indicated on the nodes of the trees.
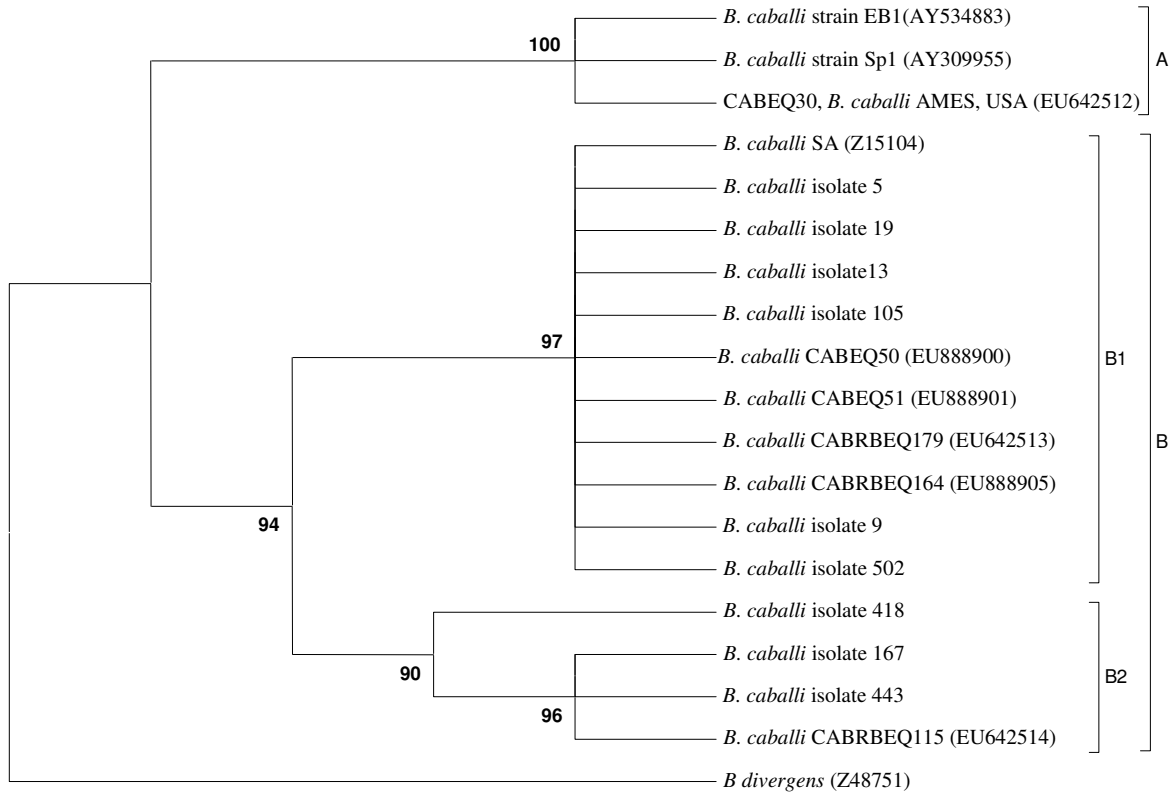
Figure 6.5 Rooted 50% majority-rule consensus cladogram, calculated using maximum-parsimony and bootstrap estimates from 1000 replicates, of the *B. caballi* 18S rRNA gene sequences identified in this study and the *B. caballi* 18S sequences from the public sequence databases (accession numbers are indicated in parenthesis). The *B. divergens* 18 rRNA gene sequence (accession number: Z48751) was used as an outgroup. Bootstrap support values are indicated on the nodes of the trees.

6.5 Discussion

Monoclonal antibody technology coupled with cELISA has been used to develop specific and sensitive serological assays for the detection of antibody to a number of infectious agents, including *B. caballi* (Knowles, 1996). A cELISA that was developed using a monoclonal antibody to RAP-1, proved to be effective in the detection of *B. caballi* antibody in the sera of infected horses from 21 different countries in the Americas and Europe (Shkap et al., 1998; Kappmeyer et al., 1999; Sevinc et al., 2008). However, in our hands the commercial cELISA kit was unable to detect *B. caballi* antibody in infected horses in South Africa, although *B. caballi* antibody was detected in 63 samples using the IFAT. Sera collected from horses during the early stages of infection and prior to the development of antibody, may result in false-negative cELISA results (Kappmeyer et al., 1999). However, this is unlikely to explain the failure of the

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cELISA to detect *B. caballi* antibody in all 63 IFAT positive samples. We therefore characterized the gene encoding RAP-1 from ten South African *B. caballi* positive *in vitro*-cultured isolates, in an attempt to explain the failure of the assay.

Two of the three primer sets which were designed based on two published *B. caballi rap-1* nucleotide sequences, AF092736 (Kappmeyer et al., 1999) and AB017700 (Ikadai et al., 1999), obtained from the USDA *B. caballi* strain, failed to amplify the homologous *rap-1* gene and flanking regions (~1800bp) from the ten South African *B. caballi in vitro*-cultured samples. Sequence analysis of the one *rap-1* fragment obtained (~615 bp) revealed sequence heterogeneity in the regions where the PCR primers had been designed, suggesting that even greater sequence heterogeneity present in the middle and at the 3' end of the *rap-1* gene in the South African isolates may have prevented the downstream amplification primers from binding to the template DNA. The PCR-based genome walking method (Rishi et al., 2004) was therefore used to amplify and sequence the unknown flanking regions of the *rap-1* fragment. This technique is commonly used to isolate upstream regions of known DNA sequences (Rishi et al., 2004), and has been successfully applied in the amplification and sequencing of a number of genes from various tick-borne pathogens that include *Ehrlichia canis* (McBride et al., 1999), *Ehrlichia ruminantium* (van Heerden et al., 2004), *Anaplasma phagocytophilum* (Zhi et al., 2002) and *Theileria parva* (Sohanpal et al., 2000). Similarly, we were able to obtain *rap-1* gene sequences from *Pvu*II digested genomic DNA of *B. caballi* isolates Bcab9 and Bcab13. An alignment of the two new sequences allowed us to design primers to amplify and sequence the *rap-1* gene from an additional eight South African *B. caballi* isolates. A nucleotide sequence alignment of all *rap-1* sequences obtained in this study and the published sequences AF092736 and AB017700, confirmed the existence of nucleotide heterogeneity in the regions where the amplification and sequencing primers had originally been designed.

Phylogenetic analyses of predicted RAP-1 amino acid sequences showed that the South African *B. caballi* sequences grouped separately from the RAP-1 amino acid sequences obtained from the USDA *B. caballi* strain (Ikadai et al., 1999; Kappmeyer et al., 1999). In addition, the South African *B. caballi* RAP-1 amino acid sequences could be further divided into two subgroups. Subgroup B1 contained RAP-1 sequences from six isolates, which showed an average of 63.4% amino acid identity to *B. caballi* RAP-1 amino acid sequences AF092736 and AB017700, while the four sequences in subgroup B2 had an average of 59.3% amino acid identity to these sequences. The groupings of RAP-1 sequences from the South African *B. caballi* isolates were

consistent with the clades observed by phylogenetic analysis of the 18S rRNA gene. This could indicate the occurrence of two antigenically different *B. caballi* strains in South Africa, which are distinct from those occurring in other parts of the world. It would be interesting to determine whether there is an association between the different *B. caballi* genetic groups identified and the clinical manifestation of equine piroplasmosis.

The testing of antigens for use in ELISAs, based on the apical complex surface-exposed epitopes, is done on the assumption that these epitopes are widely conserved among isolates (Madruga et al., 1996). The 48-kDa immunodominant rhoptry protein isolated from a USDA strain of *B. caballi* has been shown to be present in both European and South American strains of *B. caballi* (Böse et al., 1994; Böse and Hentrich, 1994; Ikadai et al., 1999). Characterization of the 48-kDa rhoptry protein indicated the occurrence of highly conserved sequences also present in the Bv60/p58 family of rhoptry genes (*rap-1* gene family) isolated from *B. bovis* and *B. bigemina* (Ikadai et al., 1999). Significant homology has been found in the nucleotide and amino acid sequences of members of the *rap-1* gene family, but sequence identity is limited to short oligopeptides, with marked sequence variation occurring in other parts of the molecule (Palmer and McElwain, 1995). While the 58 kDa RAP-1 protein of *B. bigemina* and the 60 kDa RAP-1 protein of *B. bovis* have been shown to be antigenically conserved among all the geographic strains tested (McElwain et al., 1987; Palmer et al., 1991; Suarez et al., 1994), genomic sequence polymorphism occurs in *rap-1* genes among antigenically different strains of both *B. bigemina* and *B. bovis* (Suarez et al., 1994). Four different variants of the *B. bigemina* RAP-1 protein have been identified in a biological clone (Mishra et al., 1991; Mishra et al., 1992); the amino acid sequences of these variants were highly conserved in the central region, but the sequences at the amino- and carboxy-termini of the protein were found to be variable.

The monoclonal antibody (MAb 79/17.18.5) used in the *B. caballi*-specific cELISA has been shown to bind to a peptide epitope within the carboxy-terminal repeat region of the *B. caballi* RAP-1 amino acid sequence (Kappmeyer et al., 1999). Tandemly repeated amino acid residues occurring in the carboxy-terminal region of the RAP-1 protein were hypothesized to be surface-exposed merozoite epitopes that could be used to potentially block infectivity for host erythrocytes (Suarez et al., 1991). Close inspection of the carboxy-terminal region of the RAP-1 amino acid sequences obtained from the South African *B. caballi* isolates clearly indicates marked amino acid sequence differences in this repeat region and thus the probable absence of the monoclonal antibody binding site. This observation probably explains the failure of the

cELISA to detect antibody to *B. caballi* in sera of infected horses in South Africa. It is therefore likely that variation in RAP-1 sequences in South African *B. caballi* isolates is immunologically significant, as has been shown for *B. bigemina* (Hötzel et al., 1997). RAP-1 of *B. bigemina* has two regions of sequence dimorphism at the carboxy- and amino-terminal ends, respectively. Antibodies recognizing surface-exposed B-cell epitopes present in the amino-terminal variant type 1 (NT-1) do not cross react with NT-2, and CD4 T-cells recognizing epitopes in the carboxy-terminal variant type 1 (CT-1) do not recognise CT-2 (Hötzel et al., 1997).

In the development and testing of the cELISA assay for *B. caballi*, discrepancies between CFT and cELISA results were reported for five samples, each of which originated from a different country (Kappmeyer et al., 1999). Four of these five CFT positive but cELISA negative samples were confirmed to be true positives by IFAT. The observed discrepancy was thought to be due to the early sampling of sera from recently infected horses and it was suggested that, since the sera did not originate from one place, the difference in results was not due to a lack in conservation of the carboxy-terminal repeat region. In the light of our results, however, it may be possible that the latter was indeed the case.

In this study we have shown significant heterogeneity in *rap-1* gene sequences from South African *B. caballi* isolates, which is reflected in the predicted amino acid sequences, particularly in the carboxy-terminal regions. We were able to identify two distinct groups of novel RAP-1 amino acid sequences occurring among ten South African isolates. Examination of a larger number of *B. caballi* isolates from different geographical locations around South Africa, and other countries, may lead to the identification of additional, as yet unidentified RAP-1 sequences. Such variation complicates the development of sensitive and reliable serological and molecular diagnostic assays for the detection of all *B. caballi* infections. We therefore recommend that the cELISA for the detection of antibodies to *B. caballi* should be redesigned using a monoclonal antibody that binds to a more conserved epitope of the RAP-1 antigen, or alternatively by choosing a more conserved antigenic target protein.

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CHAPTER 7
General Discussion

In most organisms, nuclear ribosomal RNA (rRNA) genes are thought to be under tight structural and functional constraint resulting in lower substitution rates and the absence of lateral gene transfer across lineages (Allsopp and Allsopp, 2006). In this study, unexpected sequence heterogeneity was found within the 18S rRNA genes of *Theileria equi* and *Babesia caballi* parasites, which led to the identification of three distinct *T. equi* and two *B. caballi* genetic groups in South African equids (Bhoora et al., 2009a; Chapter 2). Divergence from the concerted evolution model has been identified in *Plasmodium* species, which have functionally distinct rRNA 'types' that differ in their expression frequencies (Gunderson et al., 1987). Similarly, *B. bigemina* and *B. bovis* parasites have been reported to contain distinct rRNA genes, which are preferentially expressed depending on the environmental and/or developmental conditions (Reddy et al., 1991; Brayton et al., 2007; Laughery et al., 2009). Sequence heterogeneity in the 18S rRNA gene has also been reported within some species of protozoa and genetically distinct *T. equi* and *B. caballi* genotypes infecting Spanish horses have also been identified previously (Criado-Fornelio et al., 2004; Nagore et al., 2004). Although piroplasm infections in zebra were first described at the turn of the century, the molecular epidemiology and possible influence that their existence may have had on horse piroplasms has largely been overlooked. In this study, the molecular epidemiology of *T. equi*-like piroplasms of zebra was also examined with the view to further elucidating the genetic variation of *T. equi* parasites in South Africa (Chapter 4).

Sequence analysis of both horse and zebra parasites confirmed the existence of as many as 25 distinct 18S rRNA sequences for *T. equi*, which belonged to three main groups (Bhoora et al., 2009a; Chapter 2; Chapter 4). Group A predominantly contained *T. equi* 18S rRNA sequences from horses, while group B, which was shown to contain a *T. equi*-like 18S rRNA sequence from a horse in Spain (Nagore et al., 2004), thus far only contains *T. equi* 18S sequences from zebra in South Africa. Novel *T. equi* 18S rRNA sequences from both zebra and horses in South Africa grouped together in the third group, group C. These results suggest that (a) there exists an assortment of *T. equi* 18S genotypes in the equid species investigated and (b) there may be more variation in the *T. equi* genotypes in South African equids than has been previously reported. *Babesia caballi* occurred at extremely low parasitaemias in zebra and we were thus unable to draw conclusions regarding the extent of sequence heterogeneity amongst these parasites infecting zebra (Chapter 4). Although less variation was observed amongst the *B. caballi* samples from horses, six different *B. caballi* 18S sequences which formed two distinct genetic groups (A and B), were identified (Bhoora et al., 2009a; Chapter 2). Group B could be further

subdivided into subgroups B1 and B2. Taken together, these observations explained the failure of many previously reported molecular assays that target the 18S rRNA gene (Bashiruddin et al., 1999; Rampersad et al., 2003; Nagore et al., 2004) to detect both *T. equi* and *B. caballi* in South Africa.

The molecular epidemiological study of piroplasms infecting zebra revealed the existence of a *T. equi*-like genotype in zebra, which has thus far not been identified in South African horse samples. It would be interesting to examine more samples collected from horses as well as from each of the three zebra species that live in sub-Saharan Africa to determine whether the *T. equi*-like parasite identified in zebra in this study is indeed exclusive to zebra in Africa. In addition, we have not examined any samples from donkeys or mules in South Africa. It would be interesting to determine the distribution of *T. equi* and *B. caballi* genotypes in these equids as well.

Accurate diagnosis of equine piroplasmosis is essential for effective control measures. It is therefore imperative that reliable and accurate molecular diagnostic assays be developed. A conserved region outside of the V4 hypervariable region of the 18S rRNA gene has been identified previously and targeted in the development of a quantitative TaqMan real-time PCR (qPCR) assay for the detection of *T. equi* (Kim et al., 2008). Despite the existence of extensive sequence variation in South African *T. equi* 18S rRNA gene sequences, an evaluation of this assay on South African isolates indicated that it is both sensitive and specific (Bhoora et al., 2009b; Chapter 3). The *T. equi*-specific qPCR assay was further shown to be able to detect DNA in samples representative of each of the three previously identified *T. equi* 18S rRNA genotypes (groups A, B and C) (Bhoora et al., 2009b; Chapter 3). The development of an 18S rRNA-based TaqMan MGB™ qPCR assay for the detection of *B. caballi* infections in equine blood samples is described in chapter 3; this test was both sensitive and specific. Previous assays proved to be limited in their ability to detect *B. caballi* infections in field blood samples due to extremely low parasitaemias that rarely exceed 1% (Hanafusa et al., 1998) or the complete absence of circulating parasites in the blood of carrier animals (Frerichs et al., 1969; Holman et al., 1993). In addition, the *B. caballi*-specific TaqMan MGB™ qPCR assay developed in this study could detect parasite DNA from samples representative of each of the previously identified *B. caballi* 18S rRNA genotypes (A, B1 and B2).

Due to the lack of sensitivity of many of the molecular diagnostic assays used previously, the prevalence of equine piroplasmosis in South African equids has probably been under-reported. This was clearly shown for zebra samples (Chapter 4), in which only 35% of samples tested were positive for *T. equi* and *B. caballi* parasites using the reverse line blot (RLB) hybridization assay, whereas 87% were positive using the qPCR assays. The application of the *B. caballi*-specific TaqMan MGB™ qPCR assay in conjunction with the *T. equi*-specific qPCR assay enabled the rapid and accurate quantification of both *B. caballi* and *T. equi* from sub-clinically infected or carrier animals (Bhoora et al., 2009b; Chapter 3) and also provided a more accurate report on the molecular epidemiology of piroplasms infecting zebra in South Africa (Chapter 4). The *B. caballi*-specific TaqMan MGB™ qPCR assay detected as low as 1.14×10^{-4} % PE, while the detection limit of the *T. equi*-specific qPCR assay was 1.9×10^{-4} % PE. Although an evaluation of these assays on South African isolates has indicated that they are both sensitive and specific, we cannot rule out the possible existence of as yet undetected *T. equi* and *B. caballi* 18S rRNA gene sequence variants, which may compromise the sensitivities of these assays. It might therefore be advantageous to incorporate additional *T. equi*-specific and *B. caballi*-specifc qPCR assays, which target other genes, in a multiplex qPCR assay for the detection of equine piroplasmosis. To this end, we examined the ability of a previously developed *T. equi ema-1*-specific qPCR assay (Ueti et al., 2003) to detect parasite DNA in South African field samples which were shown to be positive by other tests including cELISA, IFAT, *in vitro* culture and *T. equi*-specific 18S qPCR. However, this qPCR was unable to detect parasite DNA in many *T. equi*-positive field samples. Sequence analysis of the *T. equi ema-1* gene from South African field samples and isolates confirmed the existence of distinct *ema-1* sequences, which belonged to three main phylogenetic groups. An alignment of the South African *ema-1* gene sequences indicated that the qPCR primers and probe sequence regions were conserved among all isolates occurring in group A, but nucleotide differences in sequences occurring in groups B and C explained either the failure or the reduced sensitivity of the Ueti *ema-1* TaqMan qPCR assay in detecting *T. equi* in some samples. We therefore developed and evaluated a second TaqMan MGB™ qPCR assay targeting the *T. equi ema-1* gene. Again, this assay was shown to be both sensitive and specific in the detection of *T. equi* infections (Chapter 5).

Currently, serological assays prescribed for equine piroplasmosis, which include the use of the indirect fluorescent antibody test (IFAT) and the enzyme-linked immunosorbent assay (ELISA), are restricted by antibody detection limits and cross-reactivity and have been shown to have a low sensitivity for detecting latent infections (Donnelly et al., 1980; Tenter and Friedhoff, 1986;

Weiland, 1986; Böse et al., 1995; Anonymous, 2008). Recombinant *T. equi* and *B. caballi* merozoite surface proteins, implicated in the pathogenesis of haemoprotozoan diseases, have recently been used, together with monoclonal antibodies to immunogenic epitopes on these proteins, in the development of competitive inhibition ELISAs (cELISA) (Knowles et al., 1991; Sam-Yellowe, 1996). In chapters 5 and 6, we determined whether the commercially available cELISAs were able to detect antibody to *T. equi* and *B. caballi* in the sera of infected horses from South Africa. The recombinant equi merozoite antigen (EMA-1) used in the development of the cELISA, has been shown previously to be conserved among geographically distant *T. equi* isolates (Xuan et al., 2001) and orthologous gene sequences have also been described for a number of other *Theileria* species (Katende et al., 1990; Kawazu et al., 1992; Matsuba et al., 1995; Shiels et al., 1995; d'Oliveira et al., 1996). In this study, the cELISA detected antibody to *T. equi* in the sera of infected horses from South Africa, although some discrepencies between the IFAT and cELISA alluded to the fact that there may be heterogeneity in the EMA-1 epitopes of some South African *T. equi* isolates (Chapter 5). In contrast, the cELISA developed using a monoclonal antibody to a recombinant apical complex rhoptry-associated protein (RAP-1), and which has been reported to successfully detect *B. caballi* antibody in the sera of infected horses from North and South America and various European countries (Kappmeyer et al., 1999; Sevinc et al., 2008), failed to detect *B. caballi* antibody in the sera of infected horses in South Africa (Bhoora et al., 2010; Chapter 6). In chapter 6 we looked at RAP-1 amino sequences from South African *B. caballi* isolates to ascertain the reasons for the failure of the cELISA.

Due to the extent of sequence variation in the *rap-1* gene from South African *B. caballi* isolates, a PCR-based genome walking method had to be used in order to amplify the complete open reading frame (1479 bp) encoding the RAP-1 protein (493 amino acids). Phylogenetic analyses confirmed the existence of two RAP-1 groups, with South African *B. caballi* RAP-1 amino acid sequences grouping separately from other published RAP-1 amino acid sequences (Bhoora et al., 2010; Chapter 6). Sequences from the two South African RAP-1 groups showed an average of 59.3 and 63.4% amino acid identities to published *B. caballi* RAP-1 sequences. Marked amino acid sequence differences in the carboxy-terminal repeat region of the *B. caballi* RAP-1 amino acid sequence in South African isolates, and thus the possible absence of the monoclonal antibody binding site, probably explain the failure of the cELISA to detect *B. caballi* antibody in infected horses in South Africa (Bhoora et al., 2010; Chapter 6). Since these results are based on analysis of a small sample population, examination of a larger number of *B. caballi* isolates from different geographical locations around South Africa and the rest of the world may lead to the

identification of additional RAP-1 amino acid sequences, the occurrence of which could further complicate the serological diagnosis of equine piroplasmosis. Based on our findings in this chapter, it is evident that the current cELISA developed for the detection of *B. caballi* antibody cannot be implemented as a prescribed serological assay for the diagnosis of equine piroplasmosis.

A comparison between the *T. equi* 18S rRNA and *ema-1* gene sequences obtained from the same samples showed inconsistencies between the phylogenetic groupings obtained, which precludes the possibility that the three *T. equi* 18S groupings identified represent different strains or lineages (Chapter 5). In contrast, the groupings of the *rap-1* sequences from South African *B. caballi* isolates were consistent with the clades observed by phylogenetic analysis of the 18S rRNA gene, indicating the potential occurrence of two antigenically different *B. caballi* strains in South Africa, which are distinct from those occurring in other parts of the world (Bhoora et al., 2010; Chapter 6). The results therefore suggest that *T. equi* and *B. caballi* parasites in South Africa have developed distinct evolutionary patterns. However, based on these observations, it also becomes evident that it is not possible to use the 18S rRNA gene to decide whether variants represent new species or subspecies. On the other hand, parasite outer membrane protein gene sequences, which are likely to be under intense selection pressure and therefore evolve at a more rapid rate than core function genes, will probably not give reliable phylogenetic information at the species level (Allsopp and Allsopp, 2006). Given the variation, particularly in *T. equi* in South Africa, sequence data obtained from other regions such as the ribosomal internal transcriber spacer (ITS) or the beta-tubulin gene may allow for better delineation of species and subspecies.

Owing to the extent of variation observed both within the *T. equi* 18S rRNA sequences as well as within the *B. caballi* RAP-1 and *T. equi* EMA-1 amino acid sequences, we cannot overlook the fact that there may be more variation in the 18S rRNA gene than we have already identified. Therefore, although the qPCR assays developed in this study were able to detect all currently known *T. equi* and *B. caballi* 18S rRNA sequence variants in South Africa, it is recommended that a multiplex qPCR assay, including a "catch-all" TaqMan probe similar to the *Theileria/Babesia* genus-specific probe used in the RLB, should be developed so as to ensure that if a piroplasm parasite with a novel 18S rRNA gene sequence is present, it will at least be detected by the genus-specific probe.

Another recommendation would be to evaluate the ability of the recently developed *B. caballi rap-1*-specific qPCR assay (Heim et al., 2007) in detecting parasite DNA in South African *B. caballi* isolates. Inspection of the *rap-1* nucleotide sequences from South African *B. caballi* isolates indicates the occurrence of variation in the qPCR primer and probe regions, however, which could potentially compromise the sensitivity of the *rap-1* qPCR assay. Since we have already established that the sensitivity of the *ema-1* qPCR assay developed by Ueti et al. (2003) is compromised by nucleotide sequence variation in the *ema-1* gene of South African *T. equi* parasites, the multiplex qPCR assay described by Heim et al. (2007), which employs the Ueti et al. (2003) *ema-1* qPCR assay, cannot be used to detect equine piroplasmosis in South Africa. It should be noted that the variation observed in the RAP-1 amino acid sequences in South African *B. caballi* isolates is based on data obtained from a relatively small number of samples, and, as mentioned above, investigation of a larger number of samples may reveal the existence of other RAP-1 amino acid sequences not identified in this study. Once the extent of sequence variation in the *rap-1* gene has been more thoroughly examined, a qPCR assay targeting a more conserved region of the *rap-1* gene could be developed and incorporated together with the *T. equi ema-1*-specific TaqMan MGB™ qPCR assay (Chapter 5), the *T. equi*-specific 18S rRNA qPCR assay (Kim et al., 2008) and the *B. caballi* TaqMan MGB™ qPCR assay (Bhoora et al., 2009b; Chapter 3) in a multiplex qPCR assay for the definitive detection of equine piroplasmosis.

Finally, we propose that a study be conducted to determine whether there is an association between the different *T. equi* and *B. caballi* genotypes identified in this study and disease phenotypes. A study conducted on the *B. rossi BrEMA-1* gene led to the identification of 13 *B. rossi* genotypes, four of which could be associated with complicated canine babesosis (Matjila et al., 2009). The identification of pathogenic *T. equi* strains or variants could be useful in the development of suitable control strategies for the parasite.

Equine piroplasmosis has a substantial impact on the international transport of horses, as positive animals are generally not permitted to enter piroplasm-free countries. Currently, regulations require the serological testing of horses by means of the IFAT or the ELISA to confirm seronegativity and to identify seropositive horses whose movement is restricted (Böse et al., 1995; Brüning, 1996). Due to the lack of sensitivity of these assays, however, the possible introduction of false-negative and therefore inapparent carrier animals into areas where competent tick vectors are prevalent can lead to an epizootic spread of the disease (Anonymous; 2008).

The findings in this thesis clearly highlight the challenges involved in developing rapid, sensitive and specific diagnostic assays for piroplasm parasites. Despite these challenges, we were able to develop three real-time PCR assays, which have significantly improved the sensitivity and specificity of detection of *T. equi* and *B. caballi* infections in South African horses over the currently prescribed serological assays.

Although the molecular epidemiology of equine piroplasmosis in South Africa has been studied previously (Posnett and Ambrosio, 1989; Posnett et al., 1991), results were based on the detection of parasites using DNA probe technology. This study therefore represents the first comprehensive molecular study, employing DNA sequence analysis and more advanced technologies, including real-time PCR, in the detection of equine piroplasmosis in South Africa. Our results will add to the existing knowledge of equine piroplasmosis worldwide and will be invaluable in the development of further molecular or serological diagnostic assays and in the development and implementation of successful control strategies. It is envisaged that, once validated, the possible incorporation of the real-time PCR assays developed in this study as prescribed tests by the World Organization for Animal Health (OIE), may assist in the accurate identification of carrier animals and therefore provide greater control over the spread of equine piroplasmosis globally.

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