CHAPTER 3

Development and evaluation of real-time PCR assays for the quantitative detection of *Babesia caballi* and *Theileria equi* infections in horses from South Africa

3.1 Abstract

A quantitative real-time polymerase chain reaction (qPCR) assay using a TaqMan minor groove binder (MGB™) probe was developed for the detection of *Babesia caballi* infection in equids from South Africa. Nine previously published sequences of the V4 hypervariable region of the *B. caballi* 18S rRNA gene were used to design primers and probes to target unique, conserved regions. The *B. caballi* TaqMan MGB™ qPCR assay was shown to be efficient and specific. The detection limit, defined as the concentration at which 95% of positive samples can be detected, was determined to be 1.14 x 10^{-4}% parasitized erythrocytes (PE). We further evaluated a previously reported *Theileria equi*-specific qPCR assay and showed that it was able to detect the 12 *T. equi* 18S rRNA sequence variants previously identified in South Africa. Both qPCR assays were tested on samples from two ponies experimentally infected with either *T. equi* or *B. caballi*. The qPCR assays were more sensitive than the indirect fluorescent antibody test (IFAT) and the reverse-line blot (RLB) during the early onset of the disease. The assays were subsequently tested on field samples collected from 41 horses, resident on three stud farms in the Northern Cape Province, South Africa. The IFAT detected circulating *T. equi* and *B. caballi* antibody in, respectively, 83% and 70% of the samples. The RLB detected *T. equi* parasite DNA in 73% of the samples, but none of the samples was positive for *B. caballi*, although 19 *T. equi*-positive samples also hybridized to the *Babesia* genus-specific probe. This could indicate a mixed *T. equi* and *B. caballi* infection in these samples, with either the *B. caballi* parasitaemia at a level below the detection limit of the *B. caballi* RLB probe, or the occurrence of a novel *Babesia* genotype or species. In contrast, the qPCR assays correlated fairly well with the IFAT. The *B. caballi* TaqMan MGB™ qPCR assay detected *B. caballi* parasite DNA in 78% of the samples. The *T. equi*-specific qPCR assay detected *T. equi* DNA in 80% of the samples. These results suggest that the qPCR assays are more sensitive than the RLB assay for the detection of *T. equi* and *B. caballi* infections in field samples.

3.2 Introduction

Babesia caballi and *Theileria equi* are haemoprotozoan parasites that cause equine piroplasmosis (Mehlhorn and Schein, 1998). The disease is of worldwide importance and occurs throughout the tropical and subtropical parts of the world with its prevalence being related to the distribution of its tick vectors (de Waal, 1992). Fourteen species of ixodid ticks of the genera *Dermacentor*, *Hyalomma* and *Rhipicephalus* have been identified worldwide as vectors of either *T. equi* or *B. caballi* (de Waal, 1992).

Both parasites cause disease in equids, which may be either acute or chronic with mortalities ranging from less than 10% up to 50%. The disease is generally characterized by fever and anaemia. The clinical signs are often variable and non-specific, making it easy to confuse the disease with other conditions, therefore complicating diagnosis. It is also not possible to differentiate between *B. caballi* and *T. equi* infections based on clinical signs alone. Once infected, horses may remain life-long carriers of *T. equi* infections whereas with *B. caballi* infections, which are self-limiting, horses remain carriers for up to four years (de Waal and van Heerden, 2004).

The global transport of horses has led to the spread of equine piroplasmosis from its endemic tropical and subtropical zones to more temperate regions. Stringent regulatory import restrictions are in place in some countries to prevent the entrance of horses that are carriers of *B. caballi* and *T. equi* as they may act as reservoirs of infection (Friedhoff et al., 1990; Sluyter, 2001). Regulations often require the serological testing of horses in order to confirm seronegativity and to identify seropositive animals whose movement is restricted (Böse et al., 1995; Brüning, 1996). A variety of serological methods, which include the complement fixation (CF) test, the indirect fluorescent antibody test (IFAT) and the competitive-inhibition ELISA (cELISA), have been developed for the detection of specific antibodies (Donnelly et al., 1982; Weiland et al., 1984; Knowles et al., 1991; Böse et al., 1995; Brüning et al., 1997; Kappmeyer et al., 1999). The reliability of these serological assays is, however, restricted by antibody detection limits and cross-reactivity (Brüning et al., 1997).

Molecular-based diagnostic tests, which have higher sensitivities and specificities than serological tests, and which can detect samples with very low parasitaemias, have been developed. *Babesia caballi*- and *T. equi*-specific oligonucleotide probes based on sequence differences in the small subunit (18S) ribosomal RNA (rRNA) genes (Allsopp et al., 1994) were used in a preliminary study to demonstrate transplacental transmission of *T. equi* (Lewis et al., 1999). Further developments using 18S rRNA genes as target sequences include species-specific nested polymerase chain reaction (PCR) assays (Bashiruddin et al., 1999; Rampersad et al., 2003) and the reverse-line blot (RLB) assay, which allows for the identification of novel genotypes or species and also allows for the detection of mixed infections (Gubbels et al., 1999; Nagore et al., 2004; Bhoora et al., 2009; Chapter 2).

Several parasite outer membrane protein gene sequences have also been targeted in the development of molecular diagnostic assays for equine piroplasmosis (Nicolaiewsky et al., 2001; Ueti et al., 2003; Alhassan et al., 2005; Alhassan et al., 2007; Heim et al., 2007). A quantitative real-time polymerase chain reaction (qPCR) assay targeting the gene (*ema-1*) encoding the equi merozoite antigen-1 (EMA-1) was developed to determine the number of *T. equi* parasites in the mammalian host and in *Rhipicephalus (Boophilus) microplus* ticks (Ueti et al., 2003). A multiplex assay, using the *ema-1* qPCR assay and a qPCR based on the rhoptry-associated protein (BC 48) gene of *B. caballi*, was subsequently developed and used to determine the prevalence of both *T. equi* and *B. caballi* parasites in horses in Brazil (Heim et al., 2007).

A qPCR assay, based on the 18S rRNA gene, was recently developed for the detection of *T. equi* infections in horses (Kim et al., 2008). This assay proved to be highly sensitive and specific for *T. equi*, and it allowed for the simultaneous detection and quantification of *T. equi* DNA in infected equine samples. In the present study, we describe the development and application of a TaqMan minor groove binder (MGB™) qPCR assay, also based on the 18S rRNA gene, for detection of *B. caballi* in equine field blood samples. Furthermore, we evaluate the ability of the *T. equi*-specific qPCR assay to detect all *T. equi* 18S rRNA variants that have been shown to occur in South Africa (Bhoora et al., 2009; Chapter 2).

3.3 Materials and Methods

3.3.1 In vitro culture of South African B. caballi and T. equi isolates

The South African *B. caballi* 502 culture was initiated from blood samples collected from an infected horse at the National Yearling Sale in March 2000 (Zweygarth et al., 2002). In February 2006, blood collected from a *T. equi*-infected horse resident on a farm in Vastfontein, Onderstepoort, was used to culture the *T. equi* WL isolate used in this study. The *B. caballi* 502 and *T. equi* WL isolates were propagated in purified equine red blood cells using established culture systems previously described (Zweygarth et al., 1995; Zweygarth et al., 2002). The *in vitro* culture was performed at the Agricultural Research Council-Onderstepoort Veterinary Institute (ARC-OVI).

3.3.2 Infection trial

Experimental infections were approved by the Committee of Animal Welfare of the Faculty of Veterinary Medicine, Utrecht University. Blood stabilates of *T. equi* (strain Zaria) and the USDA strain of *B. caballi* (Kappmeyer et al., 1999) were used in the infection trial. *Theileria equi* (strain Zaria) was isolated in Nigeria in 1973. A blood stabilate was prepared from an infected horse, frozen and transported to the Faculty of Veterinary Medicine at Utrecht University in the Netherlands, where pony "Nico" was infected subcutaneously with the stabilate. Blood was collected and frozen 18 days later. Five years later (1978) Pony 187 was infected both subcutaneously and intramuscularly with the blood stabilate from pony "Nico". Blood was collected and frozen ten days post infection (p.i.) and stored in liquid nitrogen. This stabilate was used in the current investigation. A blood stabilate of the USDA strain of *B. caballi* was kindly supplied by Prof. Friedhoff from the Faculty of Veterinary Medicine, Hannover, Germany.

Two three-year-old, male Shetland ponies were housed together under tick-free conditions in the large-animal experimental facility, Utrecht University. Prior to infection both ponies were tested negative for both *T. equi* and *B. caballi* by PCR/RLB hybridization and IFAT. Before infection the ponies were immuno-suppressed by the administration of dexamethasone (Dexadresone, Intervet®, Boxmeer, The Netherlands) at a dose of 0.04 mg/kg body weight (BW) by intramuscular injection, three times: four and two days before infection and on the day of infection. Pony A, a stallion weighing 150 kg, was infected with *T. equi* (Zaria) blood stabilate by 2 ml intravenous and 2 ml subcutaneous inoculation. Pony B, a gelding weighing 104 kg, was infected with 1 ml of *B. caballi* (USDA) blood stabilate administered subcutaneously and 1 ml intravenously. The horses were monitored daily for clinical responses. General physical appearance, early morning rectal temperatures, pulse and respiratory rates were monitored, mucosal membranes were inspected and lymph nodes were checked for possible enlargement. Serum and EDTA blood samples were taken daily from the jugular vein of each pony from day 3 until day 52 p.i. Haematocrit readings were taken daily, and between 3000 and 5000 erythrocytes were examined in Giemsa-stained blood smears for the presence of parasites. Two hundred µl of blood was spotted in duplicate on FTA filter paper (Whatman®). Filter papers were sent to South Africa where DNA was extracted. On day 60 p.i. both ponies were euthanized using 20 ml pentobarbital (30%) intravenously.

3.3.3 Field samples

Serum and EDTA blood samples were collected from 41 horses of unknown piroplasm status, 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.

3.3.4 Indirect fluorescent antibody test (IFAT)

The IFAT was conducted at the ARC-OVI. A standard IFAT protocol, as described in the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2004 (Anonymous, 2008) was used; antigens were locally produced in the Netherlands or South Africa. Bound equine antibodies were detected with fluorescein isothiocyanate-conjugated rabbit anti-horse immunoglobulin (RAHo/IgG(H+L)FITC, Nordic Immunology, Tilburg, the Netherlands) and examined in a wet mount by fluorescence microscopy.

3.3.5 DNA extraction

DNA was extracted from 200 µl of *in vitro* culture material or EDTA-anticoagulated blood using the QIAamp DNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, except that DNA was eluted in 100 µl. Six round holes, 3 mm in diameter, were punched out of a single blood spot stored on FTA paper (Whatman®), and DNA was extracted using the dried blood spot protocol from the QIAamp DNA mini kit (Qiagen, Hilden, Germany).

3.3.6 PCR amplification and reverse-line blot (RLB) hybridization

Primers RLB-F2 (5'-GAC ACA GGG AGG TAG TGA CAA G-3') and RLB-R2 (5'-biotin-CTA AGA ATT TCA CCT CTG ACA GT-3'), which are specific for *Theileria* and *Babesia* species, were used to amplify the V4 hypervariable region of the parasite 18S rRNA gene, as described by Nijhof et al. (2005). Samples were subjected to RLB hybridization as described previously (Bhoora et al., 2009; Chapter 2).

3.3.7 Design of a B. caballi-specific TaqMan® MGB™ qPCR assay

Six 18S rRNA gene sequences (GenBank Accession numbers: EU642512, EU642513, EU642514, EU888900, EU888901, EU888904), obtained from *B. caballi*-positive field samples in a previous study (Bhoora et al., 2009), along with three other previously published *B. caballi* 18S rRNA sequences, Z15104 (Allsopp et al., 1994), AY309955 (Criado-Fornelio et al., 2004) and AY534883 (Nagore et al., 2004), were used to develop the assay. A TaqMan minor groove binder (MGB™) probe qPCR assay was designed using the Primer express software v2.0 (Applied Biosystems). A primer pair, [Bc_18SF402: 5'-GTA ATT GGA ATG ATG GCG ACT TAA-3' and Bc_18SR496: 5'-CGC TAT TGG AGC TGG AAT TAC C-3' (IDT)], and a TaqMan® MGB™ probe [Bc_18SP: 5'-6-FAM-CCT CGC CAG AGT AA-MGB-3' (Applied Biosystems)], were designed to amplify and detect a 95 bp fragment in the V4 hypervariable region of the 18S rRNA gene. The TaqMan MGB™ probe was labeled with the fluorescent dye 6-carboxyfluorescein at the 5′ end and a non-fluorescent quencher at the 3′ end (Applied Biosystems). The forward primer and the probe are specific for *B. caballi*, but the reverse primer is not.

Real-time quantitative PCR was performed in MicroAmp optical 96-well reaction plates using the StepOnePlus™ Real-time PCR instrument (v. 2.0, Applied Biosystems). All qPCR assays were run in a total reaction volume of 20 µl comprising 1x TaqMan Fast Universal PCR Master Mix (Applied Biosystems), 0.9 µM of each forward and reverse primer, 0.25 µM TaqMan MGB™ probe and 5 µl of target DNA. The qPCR cycling conditions were as follows: activation of the FastStart DNA polymerase at 95°C for 20 seconds, then 40 cycles of 1 second at 95 °C and 20 seconds at 60 °C.

3.3.8 Theileria equi-specific TaqMan qPCR assay

Primers Be18SF (5'-GCG GTG TTT CGG TGA TTC ATA-3') and Be18SR (5'-TGA TAG GTC AGA AAC TTG AAT GAT ACA TC-3') and the TaqMan probe, Be18SP (5'-6-VIC-AAA TTA GCG AAT CGC ATG GCT T-3'), previously designed for a *T. equi*-specific qPCR assay (Kim et al., 2008) were used. The qPCR was performed as described above.

3.3.9 *Efficiency, sensitivity and specificity of the qPCR assays*

The *in vitro*-cultured South African *B. caballi* 502 and *T. equi* WL isolates with percentage parasitized erythrocytes (PE) of approximately 8.1 (~7.29 x 10^5 parasites/µl) and 13.8 (~1.24 x 10^6 parasites/µl), respectively, were used in the generation of standard curves from which the efficiencies of the qPCR assays were determined. A ten-fold dilution series (10^0 to 10^{-7}) from each of the *in vitro*-cultured equine parasites was prepared in duplicate using a suspension of uninfected equine red blood cells. DNA was extracted from all diluted samples as described above and qPCR amplifications of both standard dilution series were repeated in triplicate and on ten separate occasions. The data generated from each of the runs were used to calculate linear regression equations of quantification cycle (C_q) (Bustin et al., 2009) against log copy number. The efficiency of each assay was determined from the regression equations. SigmaPlot® (ver. 11) was used to plot a graph from which the sensitivity by concentration, for each assay, could be determined. The estimated sensitivity and 95% confidence intervals for the true sensitivity for each group of dilutions prepared were calculated using the standard error of the estimated sensitivity of each dilution group (Sibeko et al., 2008).

The analytical specificity of each assay was evaluated using DNA extracted from other protozoal parasites expected to occur in equids, including *Trypanosoma brucei evansi*, *Trypanosoma brucei equiperdum*, *Trypanosoma vivax* and either *T. equi* for the *B. caballi* qPCR assay or *B. caballi* for the *T. equi* assay. DNA extracted from blood from a piroplasm-free horse was included in each assay as a negative control.

3.3.10 *Comparison of the qPCR assays with other tests for the detection of B. caballi and T. equi*

The qPCR assays were used to detect parasites in daily blood samples taken from two experimentally infected ponies as well as from 41 field samples of unknown piroplasm status. The results were compared with serological detection by IFAT and detection of parasites by RLB hybridization.

3.4 Results

3.4.1 Specific detection of B. caballi and T. equi using the qPCR assays

The *B. caballi* TaqMan MGB™ qPCR assay proved to be efficient in the amplification of a 95 bp fragment of the V4 hypervariable region of the 18S rRNA gene from an *in vitro*-cultured *B. caballi* isolate (Figure 3.1). No amplification signal was detected from negative control DNA extracted from blood from a horse free from piroplasms or from DNA extracted from an *in vitro*-cultured *T. equi* isolate (Figure 3.1). Furthermore, no amplification signals were observed from DNA extracted from *T. b. evansi*, *T. b. equiperdum* and *T. vivax*, other protozoal parasites expected to occur in horses (Figure 3.1).

Similarly, the *T. equi*-specific qPCR assay recently developed by Kim et al. (2008) was shown to be successful in the amplification of a fragment of the *T. equi* 18S rRNA gene from DNA extracted from an *in vitro*-cultured *T. equi* isolate (results not shown). No amplification signal was obtained from negative control DNA, DNA extracted from an *in vitro*-cultured *B. caballi* isolate, or from *T. b. evansi*, *T. b. equiperdum* and *T. vivax* DNA (results not shown). The latter result agrees with the findings of Kim et al. (2008) who showed that the *T. equi* qPCR assay did not detect *B. caballi* and *Trypanosoma evansi* DNA.

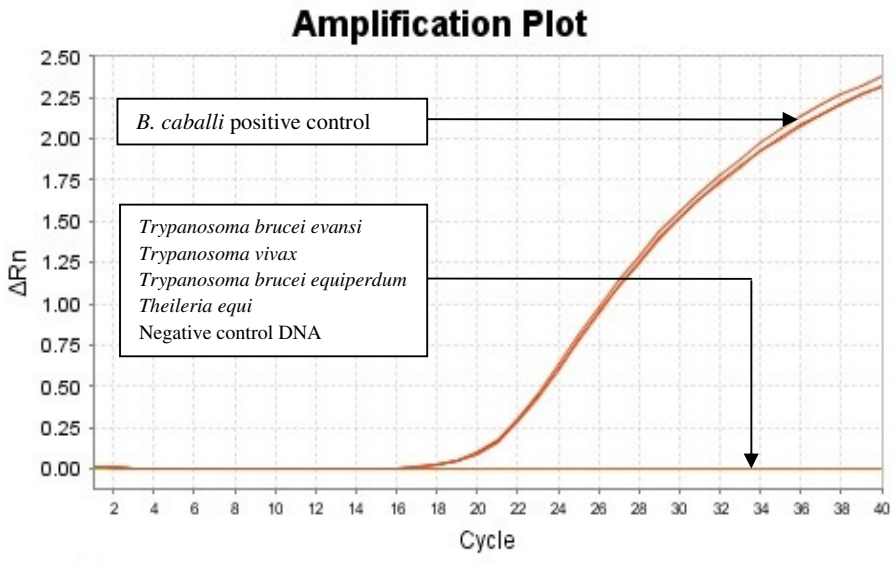
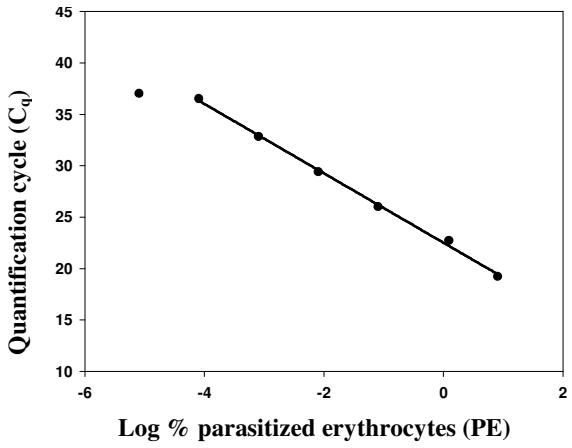


Figure 3.1 Detection of *B. caballi* positive control DNA using the *B. caballi* TaqMan MGB™ qPCR assay, indicated by an increase in the fluorescence signal. No increase in fluorescence was observed in the negative control sample (DNA extracted from blood from a piroplasm-free horse), or from *T. equi* control DNA or DNA from other protozoal parasites expected to occur in equids. The threshold (ΔRn) value was set at 0.05.

The efficiencies of the qPCR assays were determined from linear regression equations, generated from ten-fold serial dilutions of genomic DNA extracted from *B. caballi*- and *T. equi*-infected equine erythrocytes (Figure 3.2; Appendix A). For *B. caballi*, the efficiency of the TaqMan MGB™ qPCR assay was determined to be 97.8% and for the *T. equi*-specific qPCR assay, an efficiency of 99.7% was calculated.

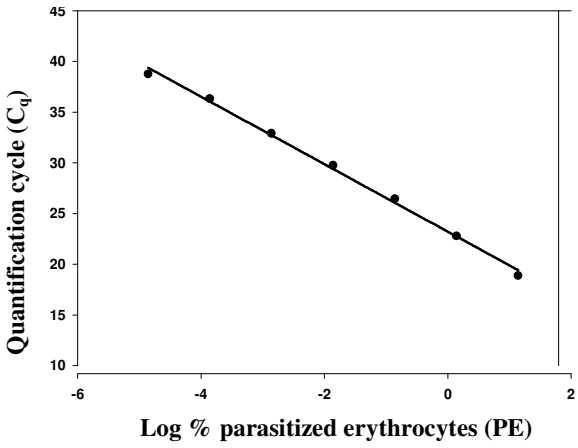
(A) *B. caballi* MGB™ qPCR standard curve



y = -3.374x + 22.52

R^2= 0.998

(B) *T. equi* MGB™ qPCR standard curve



y = -3.329x + 23.21

R^2 = 0.998

Figure 3.2 Standard curve for the quantification of **(A)** the *B. caballi* 18S rRNA gene and **(B)** the *T. equi* 18S rRNA gene. C_q values were plotted against the Log % parasitized erythrocytes (PE) of the initial ten-fold dilution series of parasite DNA equivalent to 8.1% PE to 8.1 x 10^{-7}% PE for *B. caballi* and 13.8% PE to 1.38 x 10^{-6}% PE for *T. equi*.

The qPCR assays were further tested for their ability to detect parasite DNA in field samples representative of the *T. equi* and *B. caballi* 18S rRNA genotypes previously identified in South Africa (Bhoora et al., 2009). The *B. caballi* TaqMan MGB™ qPCR assay successfully detected *B. caballi* parasite DNA in field samples representative of each of the groups of *B. caballi* 18S rRNA genotypes (groups A, B1 and B2) (Table 3.1). Similarly, the *T. equi*-specific qPCR assay could detect parasite DNA in field samples representative of each of the previously identified groups of *T. equi* 18S rRNA genotypes (groups A, B and C) (Table 3.1). Mixed infections are likely to occur in field samples and the use of the qPCR assays allowed for the identification of samples with dual *T. equi* and *B. caballi* infections.

Table 3.1 Ability of the qPCR assays to detect previously identified South African *B. caballi* and *T. equi* 18S rRNA genotypes from field samples containing single and mixed infections.

Sample name	Species and 18S rRNA genotype[a]	*B. caballi*-specific qPCR result (C_q)[b]	*T. equi*-specific qPCR result (C_q)
LFEQ23	*T. equi* group A	Negative	26.42
RBEQ32[c]	*T. equi* group A	35.85	20.88
RBEQ63[c]	*T. equi* group A	34.24	21.90
RBEQ178[c]	*T. equi* group A	39.62	28.65
RBEQ96	*T. equi* group B	Negative	23.33
RBEQ101	*T. equi* group B	Negative	24.81
EQ08	*T. equi* group C	Negative	28.73
EQ10	*T. equi* group C	Negative	29.79
EQ70	*T. equi* group C	Negative	32.38
EQ75	*T. equi* group C	Negative	32.81
LFEQ45	*T. equi* group C	Negative	35.43
LFEQ47	*T. equi* group C	Negative	36.09
LFEQ177	*T. equi* group C	Negative	33.33
RBEQ105	*T. equi* group C	Negative	27.41
RBEQ112	*T. equi* group C	Negative	31.69
CABEQ30[c]	*B. caballi* group A	18.31	34.99
CABEQ31[c]	*B. caballi* group B1	18.38	34.76
CABEQ33[c]	*B. caballi* group B1	17.37	35.11
CABEQ50	*B. caballi* group B1	17.70	Negative
CABEQ51[c]	*B. caballi* group B1	19.91	29.53
CABEQ52[c]	*B. caballi* group B1	18.52	36.78
CABEQ107[c]	*B. caballi* group B1	35.83	38.07
CABRBEQ164	*B. caballi* group B1	32.72	Negative
CABRBEQ179	*B. caballi* group B1	33.91	Negative
CABRBEQ73	*B. caballi* group B2	21.09	Negative
CABRBEQ74	*B. caballi* group B2	19.66	Negative
CABRBEQ115	*B. caballi* group B2	19.46	Negative

[a] The sequence of the 18S rRNA genotype in each sample was obtained in a previous study in which phylogenetic analyses indicated that the *T. equi* 18S rRNA sequences could be grouped into three main clades and the *B. caballi* sequences could be divided into two clades (Bhoora et al., 2009; Chapter 2).

[b] Quantification cycle value.

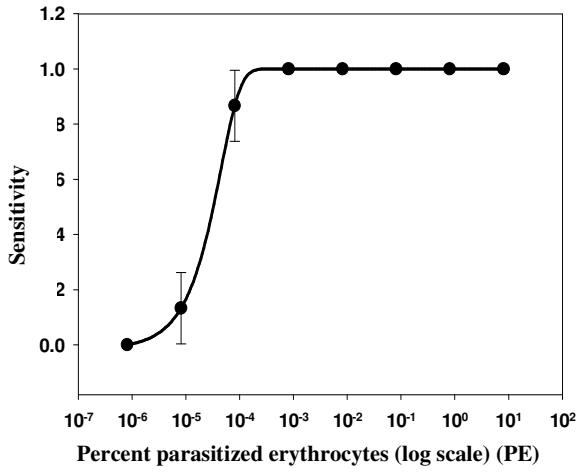
[c] Field samples with dual *T. equi* and *B. caballi* infections.

3.4.2 Analytical sensitivity of the qPCR assays

B. caballi DNA was detected in all 30 replicates of the dilutions of the cultured *B. caballi* 502 isolate ranging from undiluted (8.1% PE) to 8.1 x 10^{-4}% PE. The detection limit of the assay, defined as the concentration at which 95% of positive samples are detected (Bustin et al., 2009), was determined from the sensitivity curve (Figure 3.3A; Appendix A) to be 1.14 x 10^{-4} % PE,

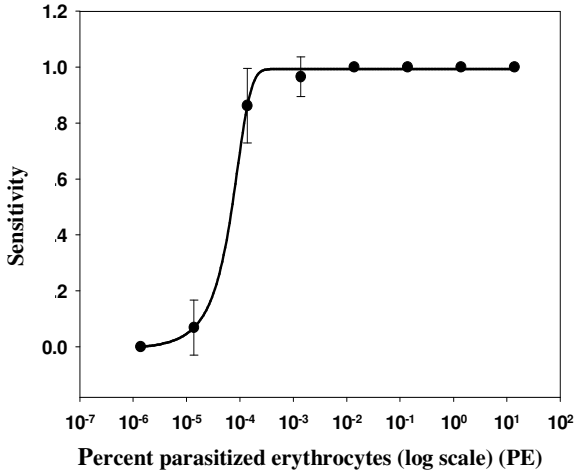
equating to a quantification cycle (C_q) of 35.82. The sensitivity of the assay decreased at higher dilutions. At 8.1 x 10^{-5}% PE, the sensitivity decreased to 86.7% with a 95% confidence interval (CI) of 74.5-98.8%, and at 8.1 x 10^{-6}% PE (C_q of 39.69), the sensitivity was 13.3% with a 95% CI of 1.16-25.4% (Figure 3A). Similarly, *T. equi* DNA was detected in all 29 replicates of the dilutions ranging from 13.8% PE to 1.38 x 10^{-2}% PE. The detection limit (95% sensitivity) of the *T. equi* qPCR assay was determined to be 1.9 x 10^{-4}% PE at a C_q value of 35.63. The sensitivity decreased from 96.5% (95% CI 89.9-100%) at 1.38 x 10^{-3}% PE (C_q of 39.38) to 6.8% (95% CI 0-16.1%) at 1.38 x 10^{-5}% PE (Figure 3.3B; Appendix A). The reportable range of qPCR assays refers to the lowest and highest results reliably produced by the test method (Sloan, 2007). For the *B. caballi*-specific MGB™ qPCR assay, C_q values greater than 39.69 were regarded as negative. Similarly, for *T. equi*, the highest reportable C_q value was determined to be 39.38.

(A) *B. caballi* MGB™ qPCR assay sensitivity



$$y = -1.6224 + \frac{2.6224}{1 + e^{\frac{-(x-(-1.507\times10^{-5}))}{3.282\times10^{-5}}}}$$

(B) *T. equi* qPCR assay sensitivity



$$y = -0.2430 + \frac{1.2361}{1 + e^{\frac{-(x-5.571\times10^{-5})}{3.359\times10^{-5}}}}$$

Figure 3.3 The sensitivity and 95% confidence intervals for the qPCR assays. **(A)** *B. caballi* TaqMan MGB™ qPCR sensitivity assay determined using a ten-fold dilution series from 10^0 to 10^{-7} prepared from a *B. caballi in vitro* culture with approximately 8.1% PE. **(B)** *T. equi* qPCR sensitivity assay determined using a 10-fold dilution series from 10^0 to 10^{-7} prepared from a *T. equi in vitro* culture with approximately 13.8% PE.

3.4.3 Comparison of the qPCR assays with other tests for the detection of B. caballi and T. equi

The newly developed *B. caballi*-specific TaqMan MGB™ qPCR assay and the previously reported *T. equi*-specific qPCR assay were compared with IFAT and RLB by testing the ability of the different tests to detect parasites or antibody in the blood of two experimentally infected ponies. Pony A, which was infected with *T. equi*, had a temperature rise (≥ 40°C), that was observed on day 8 p.i. and remained high until day 10 p.i. During this period of increased temperature the pony was lethargic, but showed no other clinical signs. On day 12 p.i. parasites were seen in blood smears but the parasitaemia never became high enough to calculate reliably (Table 3.2). From day 14 p.i. no further parasites were found in any of the blood smears. The haematocrit started to decline after infection and was less than 36% from day 6 p.i., with the lowest haematocrit value of 25% recorded on day 15 p.i. The haematocrit remained under the normal level of 36-40% until day 53 p.i. when it reached 37%. The first sample that was IFAT positive was taken on day 9 p.i. (Table 3.2) and Pony A remained IFAT positive until the end of the experiment. IFAT was not performed on all samples, since antibody titres were not expected to vary much from day to day. The first positive result of the RLB test was on day 8 p.i. and remained positive until the last test was performed on day 52 p.i., with five exceptions (days 16-18, 34 and 45) (Table 3.2). The qPCR test appeared to be the most sensitive of the three tests as it was able to detect *T. equi* in Pony A from day 7 p.i. and remained positive until day 52 p.i.

Pony B, infected with *B. caballi,* had an increased temperature (≥ 40°C) from day 6 p.i. until day 15 p.i. During this time the animal suffered from general malaise; it moved slowly, laid down frequently and had a decreased appetite. Parasites were seen in the blood smear from day 9 p.i. (Table 3.2). From day 19 p.i. no parasites were found, except for day 22 p.i. when a single parasite was seen. After infection, the haematocrit value declined to a low of 20% on day 19 p.i. and then increased again, reaching a normal haematocrit value (36%) on day 46 p.i. Pony B tested IFAT positive on day 10 p.i. and remained IFAT positive until the last test was performed on day 52 p.i. (Table 3.2). According to the RLB test, the first positive sample was on day 9 p.i., and samples remained positive until day 51 p.i. The RLB was negative on day 52 p.i. when the last test was performed (Table 3.2). The TaqMan MGB™ qPCR test was able to detect *B. caballi* from day 7 until day 52 p.i.

The PCR-based DNA detection tests used in this study were more sensitive than IFAT during the early onset of the disease, since they detected *T. equi* and *B. caballi* DNA in the circulation prior to antibody production. After day 9 p.i. for Pony A and day 10 p.i. for Pony B all three tests were in full agreement, with the exception of five days when the RLB was negative for *T. equi* (Pony A) and one day when it was negative for *B. caballi* (Pony B). On most days when the RLB was negative for *T. equi*, the qPCR C_q values were very high (Table 3.2). The variations in the qPCR C_q values over time likely reflect the concomitant fluctuations in the circulating parasitaemia. The negative RLB results were thus probably as a result of periods when the parasitaemia dropped below the detection limit of the RLB. The qPCR assays were more sensitive than the RLB as they were able to detect parasite DNA in both ponies earlier than the RLB hybridization assay, and qPCR results remained positive until the last day.

Table 3.2 Results of microscopic examination of blood smears, IFAT, RLB and qPCR tests for Pony A infected with *T. equi* and Pony B infected with *B. caballi*.

Days	Pony A (infected with *T. equi*)				Pony B (infected with *B. caballi*)			
p.i.	Blood	IFAT	RLB	*T. equi*-	Blood	IFAT	RLB	*B. caballi*-
3	-	0	-	-	-	0	-	-
4	-		-	-	-		-	-
5	-		-	-	-		-	-
6	-		-	-	-		-	-
7	-	0	-	35.11	-	0	-	36.30
8	-	0	+	34.12	-	0	-	34.43
9	-	160	+	31.89	+	0	+	31.79
10	-	640	+	30.85	+	160	+	29.63
11	-	1280	+	30.72	+	320	+	28.48
12	+		+	30.27	+		+	26.47
13	+		+	30.98	+		+	26.53
14	-		+	35.18	+		+	31.67
15	-	>2560	+	32.93	+	320	+	27.34
16	-		-	35.35	+		+	29.93
17	-	5120	-	37.77	+	2560	+	29.24
18	-		-	38.30	+		+	31.08
19	-	2560	+	35.57	-	640	+	31.17
20	-		+	32.99	-		+	31.17
21	-		+	33.04	-		+	31.16
22	-	2560	+	31.65	+	1280	+	32.85
24	-		+	31.40	-		+	31.08
26	-	>2560	+	33.62	-	640	+	30.50
28	-		+	37.32	-		+	30.50
31	-	2560	+	31.54	-	640	+	30.44
34	-		-	34.31	-		+	32.81
38	-	>2560	+	33.05	-	1280	+	32.50
45	-	>2560	-	36.17	-	1280	+	32.95
48	-	5120	+	32.39	-		+	32.76
52	-	>1280	+	33.40	-	640	-	32.75

[a] *T. equi* C_q values greater than 39.38 are considered negative.

[b] *B. caballi* C_q values greater than 39.69 are considered negative.

In order to further evaluate the *T. equi* and *B. caballi* qPCR assays, we tested 41 field samples of unknown piroplasm status (Table 3.3). Due to the low prevalence of *B. caballi* in field blood samples in South Africa, we targeted three stud farms in the Northern Cape Province, where tick vectors for both *T. equi* and *B. caballi* occur, namely, *Hyalomma truncatum* and *Rhipicephalus evertsi evertsi* (de Waal and van Heerden, 2004). Tick-control strategies were only implemented on the selected stud farms when high tick burdens were observed and therefore horses were almost certainly exposed several times to the tick vectors and parasites, making them an ideal target population for detecting natural *T. equi* and *B. caballi* infections. The *B. caballi* TaqMan MGB™ qPCR assay detected *B. caballi* parasite DNA in 32 of the 41 field samples with C_q

values that ranged between 23.62 and 37.41. The *T. equi* qPCR assay detected *T. equi* parasite DNA in 33 of the 41 samples with C_q values between 22.37 and 34.49.

The qPCR results were compared to those obtained by IFAT and RLB (Table 3.3). The IFAT detected circulating *T. equi* and *B. caballi* antibody in, respectively, 83% and 70% of the samples tested (Table 3.3). The RLB detected *T. equi* parasite DNA in 73% of samples tested, while none of the samples was positive for *B. caballi*. However, 19 of the *T. equi* positive samples also hybridized to a *Babesia* genus-specific probe. This could indicate a mixed *T. equi* and *B. caballi* infection with either the *B. caballi* parasitaemia at a level below the detection limit of the *B. caballi* RLB probe, or the occurrence of a novel *Babesia* genotype or species. In a molecular epidemiological survey of equine piroplasmosis conducted in Spain, Nagore et al. (2004) showed that of the 243 equine blood samples tested using the RLB, *B. caballi* was detected in only one sample and *B. caballi-like* parasites in a further eight samples. *Babesia caballi* infections have been reported to occur at very low parasitaemias that rarely exceed 1% (Hanafusa et al., 1998). Previous reports indicate that *B. caballi* is extremely difficult to detect in blood smears at any stage of the disease except the early acute phase, and once a carrier status is established, there may be complete absence of circulating parasites (Frerichs et al., 1969; Holman et al., 1993). The occurrence of such low parasitaemias could possibly explain the inability of the RLB to detect all positive *B. caballi* infections. Alternatively, the presence of sequence variation, which has previously been reported to occur in the region of the 18S rRNA gene where the RLB primers and probes were designed (Bhoora et al., 2009; Chapter 2), could explain the discrepant results. Despite the apparently low parasitemias, or the possible presence of sequence variants, the *B. caballi* TaqMan MGB™ qPCR assay proved to be efficient in detecting infected animals. The assay detected *B. caballi* parasite DNA in 78% of the samples tested, 34% (17) of which were from samples co-infected with *T. equi* as shown by the *T. equi*-specific qPCR assay. The *T. equi*-specific qPCR assay was also shown to be more sensitive than the RLB, and could detect parasite DNA in 80% of the samples tested.

Table 3.3 IFAT, RLB and qPCR results for 41 equine field blood samples of unknown piroplasm status, obtained from three stud farms in the Northern Cape Province, South Africa, where limited tick-control measures were implemented.

Sample no.	*T. equi* IFAT	*B. caballi* IFAT	RLB	*T. equi* qPCR (C_q)	*B. caballi* qPCR (C_q)
1	Positive	Positive	*T. equi*, B. catch all[a]	25.06	32.76
2	Positive	Positive	*T. equi*, B. catch all	25.14	32.43
3	Positive	Positive	*T. equi*	34.49	37.41
4	Positive	Positive	*T. equi*, B. catch all	24.18	32.49
5	Positive	Positive	*T. equi*	29.99	34.85
6	Positive	Positive	*T. equi*, B. catch all	25.53	32.83
7	Positive	Positive	T/B. catch all[b]	25.07	32.72
8	Positive	Positive	Negative	34.13	30.72
9	Positive	Positive	*T. equi*, B. catch all	23.86	28.43
10	Positive	Positive	*T. equi*, B. catch all	24.19	29.36
11	Positive	Positive	*T. equi*, B. catch all	Negative	Negative
12	Positive	Positive	*T. equi*, B. catch all	22.37	29.76
13	Positive	Negative	*T. equi*, B. catch all	25.09	36.69
14	Positive	Positive	*T. equi*, B. catch all	24.09	28.29
15	Positive	Positive	*T. equi*, B. catch all	24.72	31.58
16	Positive	Positive	*T. equi*, B. catch all	26.04	36.43
17	Positive	Positive	*T. equi*, B. catch all	26.18	31.70
18	Negative	Positive	Negative	30.13	37.29
19	Positive	Positive	*T. equi*, B. catch all	25.39	29.95
20	Positive	Positive	*T. equi*, B. catch all	Negative	26.41
21	Positive	Positive	*T. equi*, B. catch all	23.24	29.70
22	Positive	Positive	*T. equi*, B. catch all	25.02	36.39
23	Positive	Positive	*T. equi*	30.00	33.49
24	Positive	Positive	*T. equi*	22.87	34.84
25	Positive	Positive	*T. equi*	24.50	29.64
26	Positive	Positive	T/B. catch all	32.16	36.51
27	Positive	Negative	*T. equi*	22.48	33.90
28	Positive	Positive	*T. equi*, B. catch all	22.86	23.62
29	Positive	Positive	*T. equi*, B. catch all	31.06	Negative
30	Positive	Positive	*T. equi*	23.17	36.18
31	Negative	Negative	Negative	Negative	Negative
32	Positive	Negative	*T. equi*	29.08	Negative
33	Negative	Negative	Negative	Negative	36.53
34	Negative	Negative	Negative	Negative	Negative
35	Negative	Negative	Negative	Negative	Negative
36	Negative	Positive	Negative	Negative	Negative
37	Positive	Negative	*T. equi*	30.10	36.83
38	Positive	Negative	*T. equi*	30.43	Negative
39	Positive	Negative	*T. equi*	28.64	36.40
40	Negative	Negative	Negative	Negative	Negative
41	Positive	Negative	Negative	34.12	32.98

[a] B. catch all – *Babesia* genus-specific probe

[b] T/B catch all – *Theileria/Babesia* genus-specific probe

3.5 Discussion

In the present study we describe the development and application of a TaqMan MGB™ qPCR assay targeting the *B. caballi* 18S rRNA gene, for the quantitative detection of the parasite from equine blood samples, as well as the evaluation of a qPCR assay for the detection of *T. equi* infections in South Africa. Molecular tests previously developed for the detection and differentiation of equine parasite species were based on conventional PCR and probe-based assays, which are relatively sensitive, but involve complex procedures which are time-consuming (Allsopp et al., 1993; Bashiruddin et al., 1999; Nicolaiewsky et al., 2001; Rampersad et al., 2003; Alhassan et al., 2005). Quantitative PCR technology has recently been applied to the diagnosis of many organisms of veterinary and medical importance (Jeong et al., 2003; Lindh et al., 2007; O'Grady et al., 2008; Wengi et al., 2008). This technology provides several advantages over the use of conventional PCR assays. Detection and quantification of a PCR product takes place in a single tube during the cycling process, thus eliminating the need for post-PCR manipulation and reducing the risk of contamination. Quantitative PCR tests have been developed for a number of haemoparasitic diseases including *Theileria sergenti* (Jeong et al., 2003), *Babesia bovis* and *Babesia bigemina* (Buling et al., 2007), *Anaplasma marginale* (Carelli et al., 2007) and *Theileria parva* (Sibeko et al., 2008). These qPCR assays have significantly improved the sensitivity and specificity of parasite detection. While a qPCR assay for the detection of *T. equi* (Kim et al., 2008), based on the amplification of the 18S rRNA gene has been developed, there is no report on the application of a qPCR test for the quantitative diagnosis of equine *Babesia* parasites using this gene.

Although sequence heterogeneity has been demonstrated in the 18S rRNA gene of *B. caballi* in South Africa (Bhoora et al., 2009), we chose this gene as the target for development of a *B. caballi*-specific qPCR assay. Other genes previously targeted for the development of molecular diagnostic assays encode outer membrane proteins (Nicolaiewsky et al., 2001; Ueti et al., 2003; Alhassan et al., 2005; Alhassan et al., 2007; Heim et al., 2007) which are under intense selection pressure, and sequence heterogeneity in the genes encoding these proteins is therefore likely to be even greater than in the 18S rRNA gene. Prior to the development of the primers and probes, the *B. caballi* 18S rRNA sequence variants were carefully examined for conserved regions. TaqMan MGB™ probes have previously been demonstrated to be helpful in the case of variable nucleotide sequences, since they allow for the use of smaller probes that are capable of detecting shorter conserved regions and with lower fluorescent background signals, as the 3' end of the probe is labelled with a non-fluorescent quencher (Kutyavin et al., 2000). Therefore, for a

robust and quantitative assay, a TaqMan MGB™ probe was designed in a conserved target sequence of the V4 hypervariable region of the *B. caballi* 18S rRNA gene.

The *T. equi*-specific qPCR assay designed by Kim et al. (2008) targets a region of the 18S rRNA gene that occurs outside of the V4 hypervariable region. Inspection of the 18S rRNA sequences obtained from the twelve distinct South African *T. equi* variants (Bhoora et al., 2009; Chapter 2), indicated the occurrence of a single nucleotide difference in the forward primer target sequence and no differences in the regions where the reverse primer and probe had been designed. These differences did not appear to affect the sensitivity of the qPCR assay developed by Kim et al. (2008) as it was able to detect all *T. equi* variants thus far identified.

The *B. caballi*-specific TaqMan MGB™ qPCR assay was highly sensitive proving to be able to detect as few as 1.14 x 10^{-4}% PE. The sensitivity of the *B. caballi* TaqMan MGB™ qPCR assay is comparable to that reported for the qPCR assay developed for the detection of *T. parva* (8.79 x 10^{-4}% parasitaemia) in cattle and buffalo in South Africa (Sibeko et al., 2008). Kim et al. (2008) determined the detection limit of their *T. equi* qPCR to be 1.5 parasites per µl per sample, which equates to 1.5 x 10^{-5}% PE. In our hands, the detection limit (95% sensitivity) of the *T. equi* qPCR assay was 1.9 x 10^{-4}% PE. Both assays were shown to be specific for the target organism and no amplification signals were observed from DNA of other protozoal parasites expected to occur in equids. *Babesia caballi* infections generally tend to occur at extremely low parasitaemias, often due to the early elimination of the parasite after a short period of infection, making diagnosis almost impossible (Frerichs et al., 1969). The development of a highly sensitive and specific qPCR assay provides a major advantage in the detection of *B. caballi* infections in field blood samples.

The high seroprevalence of *T. equi* and *B. caballi* on three stud farms in the Northern Cape Province where limited tick-control measures were implemented confirmed the occurrence of both *T. equi* and *B. caballi* infections on the selected farms. Although there was significant correlation between the qPCR and IFAT results in the detection of *B. caballi* and *T. equi* in infected horses, minor differences between the results were observed. Three samples were IFAT-positive for *B. caballi* but the qPCR results were negative. In addition, *B. caballi* DNA could be detected in six samples that were reported to be *B. caballi* IFAT-negative. Similarly for *T. equi*, two IFAT-positive samples were negative when tested using the qPCR, while *T. equi* parasite DNA could be detected in one sample that was reported to be IFAT-negative.

Quantitative PCR tests detect the presence of parasite DNA, whereas IFAT detects antibodies, which can be present in the absence of parasites (Holman et al., 1993). Depending on the tick vectors, the prepatent period of *B. caballi* infections is between 10-20 days, (de Waal and Potgieter, 1987; de Waal, 1990) and that of *T. equi* infections is reported to be between 12-14 days (Mehlhorn and Schein, 1998). The sampling time thus plays a critical role in the detection of circulating parasites. The occurrence of IFAT-negative but qPCR-positive results may be explained by the observation that parasites can be detected in newly infected animals prior to the development of antibodies. This can be observed in the results obtained from our experimental infection trial (Table 3.2), where both ponies remained IFAT-negative during the first few days of infection even though parasite DNA was detected by the qPCR assays. Allsopp et al. (2007) reported the detection of *T. equi* parasite DNA in a four month old aborted foetus, which indicated that transplacental transmission takes place long before the foetal immune system is sufficiently developed to recognize these parasites as foreign. Foals born to carrier mares in endemic areas, however, usually acquire maternal antibodies in the colostrum, which can be detected up to five months of age (Donnelly et al., 1982). However, if sample collection occurred prior to colostrum ingestion, new-born naïve foals may test serologically negative, but PCR positive (Allsopp et al., 2007; Kumar et al., 2008).

Babesia caballi infections are self-limiting, usually lasting one to three years, and horses are generally able to eliminate the infection naturally or drugs can be used to sterilize the infection (Friedhoff and Soule, 1996; Brüning et al., 1996), although a recent study has shown that even high-dose treatment with imidocarb may not be capable of eliminating *B. caballi* infections from healthy carriers (Butler et al., 2008). It is however possible that *B. caballi* antibody titres remain at detectable levels for some period after the parasite has been transiently cleared, either naturally or by the use of drugs, resulting in animals testing IFAT-positive but negative using PCR-based methods. *Theileria equi* infections are not self-limiting and, once infected, horses remain life-long carriers of the parasite. Samples that test positive for *T. equi* by IFAT should therefore be detectable by qPCR, unless the parasitaemia is below the detection limit of the assay. However, IFAT-positive and qPCR-negative results could also be explained either by the existence of parasite 18S rRNA gene sequence variants that have not yet been identified and can therefore not be detected by the qPCR tests or the presence of PCR inhibitors.

To our knowledge, this is the first report on the development of a quantitative TaqMan MGB™ qPCR assay, based on the 18S rRNA gene, for the detection of *B. caballi* infections in equine blood samples. Accurate diagnosis of equine piroplasmosis is essential for effective control measures. Previous assays proved to be limited in their ability to detect *B. caballi* infections in field blood samples due to the extremely low or undetectable parasitaemias observed. We have demonstrated rapid and accurate quantification of *B. caballi* from sub-clinically infected or carrier animals, using the TaqMan MGB™ qPCR assay on a StepOnePlus real-time PCR instrument. We envisage that application of this assay, along with the *T. equi*-specific qPCR assay developed by Kim et al. (2008), will provide better confirmation of diagnosis of equine piroplasmosis, particularly in cases where symptoms are non-specific. However, while we were able to show that the *B. caballi* and *T. equi* qPCR assays were able to detect all known 18S rRNA sequence variants that have previously been identified in South Africa, we do not know whether other variants exist in the field. It would therefore be prudent to develop a multiplex qPCR assay, including a "catch-all" TaqMan probe, similar to the *Theileria/Babesia* genus-specific probe used in the RLB, to ensure that if a piroplasm parasite is present in a sample, it will be detected by the qPCR test. Once validated, the tests could be incorporated as required tests by the OIE for the import and export of horses and for checking whether attempts at sterilizing equine piroplasmosis infections have been successful.

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CHAPTER 4

Genetic diversity of piroplasms in plains zebra (*Equus quagga burchellii*) and Cape mountain zebra (*Equus zebra zebra*) in South Africa

4.1 Abstract

Seventy EDTA blood samples collected from plains zebra (*Equus quagga burchellii*) and Cape mountain zebra (*Equus zebra zebra*) were screened for the presence of piroplasm DNA using the reverse line blot (RLB) hybridization assay, and quantitative *T. equi*-specific and *B. caballi*-specific TaqMan real-time PCR (qPCR) tests. RLB results indicated that 17 samples were positive for *T. equi*, while no samples hybridized to the *B. caballi* probe. Eight samples hybridized only to the *Theileria/Babesia* genus-specific probe and 45 samples were negative for the presence of piroplasms. In contrast, the *T. equi*-specific qPCR assay detected *T. equi* parasite DNA in 59 samples and the *B. caballi*-specific qPCR was able to detect *B. caballi* parasite DNA in 19 samples. The complete 18S rRNA gene was amplified and sequenced from 17 samples, while the V4 hypervariable region of the 18S rRNA gene could be amplified, cloned and sequenced from 31 samples. BLASTN analysis revealed that all of the sequences obtained were most similar to *T. equi* genotypes and not *B. caballi* genotypes. Although *Babesia* parasites were present in some of these samples, as indicated by qPCR, the parasitaemia may have been too low to allow detection by cloning of PCR products from a mixed infection. Based on these findings and our previous results obtained for parasites from horses, we conclude that there are three groups of *T. equi* 18S rRNA sequences in South Africa, designated A, B and C. Sequence analyses of both the full-length and the V4 hypervariable region of the *T. equi* 18S rRNA gene suggest the existence of 13 new *T. equi* sequences from zebra, including a novel sequence in clade A, seven new sequences in clade B and five novel sequences in clade C. These results confirm the existence of sequence heterogeneity in the rRNA genes of the parasites that cause equine piroplasmosis, and suggest that there may be additional, as yet unidentified, *T. equi* and *B. caballi* 18S rRNA sequences present in the horse and zebra populations in South Africa. The occurrence of previously unrecognized sequence variation could pose a potential problem in the implementation of diagnostic tests targeting the 18S rRNA gene.

4.2 Introduction

Equine piroplasmosis is caused by the intra-erythrocytic, tick-transmitted protozoan parasites *Theileria equi* (Mehlhorn and Schein, 1998) and *Babesia caballi* (Nuttal and Strickland, 1910), which affect horses, mules, donkeys and zebras. Twelve species of ixodid ticks in the genera *Dermacentor, Rhipicephalus* and *Hyalomma* have been identified worldwide as vectors of the disease (de Waal and van Heerden, 2004). Piroplasmosis of horses has an economic impact on the international movement of horses from endemic to disease-free areas (de Waal, 1992).

Various serological and molecular assays have thus been developed to detect *T. equi* and *B. caballi* in infected horses and to identify and allow the movement of negative horses (Brüning et al., 1997; Avarzed et al., 1998; Ikadai et al., 2000; Nagore et al., 2004; Heim et al., 2007; Kim et al., 2008).

A recent study in our laboratory on the molecular characterization of the small subunit (18S) ribosomal RNA (rRNA) genes of equine piroplasms revealed that several molecular assays based on this gene failed to detect the parasites because of sequence variation in the target gene (Bhoora et al., 2009a; Chapter 2). Furthermore, we found that the nucleotide sequences of two zebra samples showed a high degree of identity to a previously published *T. equi*-like strain (*Babesia equi* ET1, accession number AY534884) identified from a horse in Spain (Nagore et al., 2004), which was not detected in any of the South African horse samples, suggesting that there may be more variation in *T. equi* 18S rRNA genotypes in zebra in South Africa.

The African continent is home to the largest number of species in the genus *Equus*. Three species of zebra survive in sub-Saharan Africa. Plains zebra (*Equus quagga*) are the most common and geographically widespread. Historically, the species occurred from the south of Ethiopia through East Africa to Angola and South Africa. Its range has been much reduced by human activities, but it remains common in game reserves. Six subspecies are recognized (Groves and Bell, 2004), of which Burchell's zebra (*Equus quagga burchellii*) occurs in South Africa. The Cape mountain zebra (*Equus zebra zebra*), a subspecies of mountain zebra (*Equus zebra*), once inhabited all the mountain ranges of the Western and Eastern Cape Provinces of South Africa, but today its distribution is limited to small pockets in game reserves (Penzhorn, 1988). Grévy's zebras (*Equus grevyi*) are found in Kenya and Ethiopia. Small populations of wild asses, from which donkeys are descended (Beja-Pereira et al., 2004), remain in the deserts of Ethiopia and Somalia (Blench, 2000). All of these wild equids are likely to carry piroplasms, which will have co-evolved with their hosts and vectors before the introduction of horses and donkeys into South Africa in the mid 17th century (Thom, 1952; Starkey, 2000).

Piroplasm infections in zebra were first described early in the twentieth century, from an East African plains zebra (Koch, 1905). The inoculation of zebra blood into a susceptible horse led to an increase in the animal's temperature and the appearance of parasites. These observations thus suggested that zebras are also carriers of piroplasms. *Theileria equi* parasites were subsequently found incidentally in the blood of plains zebras from Umfolozi in KwaZulu-Natal,

South Africa (Neitz, 1931) and parasites were also reported to occur in Cape mountain zebras in South Africa (Young et al., 1973; Zweygarth et al., 2002). *Babesia caballi* parasites have also been reported from both plains and Grévy's zebras in East Africa (Neitz, 1965; Zweygarth et al., 2002). Despite the knowledge that these parasites occur in our zebra populations, the molecular epidemiology and the possible influence through genetic recombination that their existence may have on horse piroplasms, which are thought to have originated in Asia (de Waal and van Heerden, 2004), has largely been overlooked. We therefore screened zebra samples to identify piroplasm parasites, and sequenced the 18S rRNA gene of *T. equi*-like piroplasms of zebra to further elucidate genetic variation in *T. equi* parasites in South Africa.

4.3 Materials and Methods

4.3.1 Samples and DNA extraction

Seventy EDTA blood samples were obtained from the Kruger National Park (n=20), the Wildlife Biological Resource Centre (n=14) and the Equine Research Centre (n= 36) (Table 4.1). These samples were collected from plains (*E. quagga burchellii*) and Cape mountain zebra (*E. zebra zebra*). Genomic DNA was extracted from 1 ml of EDTA blood using the QiaAmp Blood Midi Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Table 4.1 Origin and number of zebra samples tested.

Origin	Zebra species	Geographical location	No.
Equine Research Center, University of Pretoria	*E. quagga burchellii*	Western Cape	23
	E. quagga burchellii	Northern Cape	13
Kruger National Park	*E. quagga burchellii*	Limpopo and Mpumalanga	10
Bontebok National Park	*E. zebra zebra*	Western Cape	10
Wildlife Breeding Research Centre			
Rietfontein farm	*E. quagga burchellii*	KwaZulu-Natal	2
Atherstone Nature Reserve	*E. quagga burchellii*	North West Province	12
Total			**70**

4.3.2 PCR amplification and reverse line blot (RLB) hybridization

Primers RLB-F2 (5'-GAC ACA GGG AGG TAG TGA CAA G-3') and RLB-R2 (Biotin-5'-CTA AGA ATT TCA CCT CTA ACA GT-3') specific for *Theileria* and *Babesia* species, were used to amplify the V4 hypervariable region of the 18S rRNA genes of the parasites present in the samples as described previously (Nijhof et al., 2005). PCR products were subjected to RLB hybridization as described previously (Bhoora et al., 2009a; Chapter 2). Since the RLB is likely to result in many false-negatives, either if the parasitaemia in the sample is very low or due to the occurrence of novel genotypes, the presence of *T. equi* and *B. caballi* parasite DNA was confirmed using a *T. equi*-specific TaqMan qPCR assay (Kim et al., 2008) and a TaqMan MGB™ qPCR for *B. caballi* (Bhoora et al., 2009b; Chapter 3).

4.3.3 Cloning and sequencing

It was not possible to obtain sufficient PCR product to directly sequence the full-length 18S rRNA gene from any of the samples that were positive by RLB and/or qPCR assays, nor was there sufficient DNA to clone the amplicons. Three overlapping nested PCRs were therefore used to amplify ~1480 bp of the complete 18S rRNA gene and nested PCR products were sequenced. The Genomiphi DNA amplification kit (Amersham Biosciences) was used to exponentially amplify genomic DNA from samples with low parasitaemia, prior to performing nested PCRs for amplification and direct sequencing of the complete 18S rRNA gene as described previously (Bhoora et al., 2009a; Chapter 2). Briefly, 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') were used in a primary PCR to amplify a ~1600 bp 18S rRNA fragment, using the cycling conditions previously reported (Bhoora et al., 2009a; Chapter 2). Three nested PCR reactions were subsequently performed, using the primary amplicon as template and primer pairs NBabesia1F and BT18S3R (5'-CCT CTG ACA GTT AAA TAC GAA TGC CC-3'), BT18S2F (5'-GGG TTC GAT TCC GGA GAG GG-3') and BT18S2R (5'-CCC GTG TTG AGT CAA ATT AAG CCG-3'), and BT18S3F (5'-GGG CAT TCG TAT TTA ACT GTC AGA GG-3') and 18SRev-TB. PCR products were purified using a QIAquick® PCR Purification Kit (Qiagen) and sequenced directly using BigDye chemistry (v.3.1, Applied Biosystems) in a 3130XL sequencer (Applied Biosystems).

Nested PCR products containing the V4 hypervariable region of the 18S rRNA gene, obtained by amplification with primers BT18S2F (5'-GGG TTC GAT TCC GGA GAG GG-3') and BT18S2R (5'-CCC GTG TTG AGT CAA ATT AAG CCG-3'), were 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 in ImMedia™ Amp Liquid (Invitrogen). Plasmid DNA was isolated by means of a High Pure Plasmid Purification kit (Roche). In an attempt to minimize the effects of PCR artifacts introduced during nested PCRs, a consensus sequence, using T7 and SP6 primers, was obtained from six clones from each sample.

Sequences were assembled and edited using gap4 of the Staden software suite (Staden, 1996; Staden et al., 2000). BLASTN (Altschul et al., 1990) was used to search the public sequence databases for orthologous sequences.

4.3.4 Phylogenetic analyses

Multiple sequence alignments were performed using the Mafft sequence alignment tool (Multiple Sequence Alignment) employing the FFT-NS-1 algorithm (Katoh et al., 2002; Katoh et al., 2005) and alignments were manually edited using BioEdit version 7.0.5.2 (Hall, 1999). 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. Phylogenetic analyses using distance and maximum likelihood methods were carried out using PAUP* v4b10 (Swofford, 2003) with 1481 positions for the complete *T. equi* 18S rRNA gene and 673 positions for the V4 hypervariable region. The 18S rRNA gene sequence of *Hepatozoon canis* (DQ439543) was used as an outgroup in the construction of phylogenetic trees (1000 replicates). Analysis by Bayesian inference was performed using MrBayes v.3.1.2 (Huelsenbeck and Ronquist, 2001; 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 1,000,000 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).

4.4 Results

RLB results for the 70 zebra samples tested showed that 25 samples (35.71%) were infected with a piroplasm parasite as indicated by hybridization to the *Theileria/Babesia* genus-specific probe (Table 4.2). Of these, 17 samples (24.3%) hybridized to the *T. equi* probe while none of the samples hybridized to the *B. caballi* probe. Eight samples (11.4%) hybridized only to the *Theileria/Babesia* genus-specific probe and not to any of the species-specific probes. Forty-five samples (64.3%) tested negative on RLB for the presence of piroplasms.

A linear standard curve generated in our laboratory using a *T. equi*-specific qPCR assay developed by Kim et al. (2008) revealed that the detection limit of the TaqMan assay (95% sensitivity) was 1.9 x 10^{-4}% parasitised erythrocytes at a quantification cycle (C_q) of 35.89 (Bhoora et al., 2009b; Chapter 3). The upper limit of detection of the *T. equi*-specific qPCR, calculated from the sensitivity curve, equated to a C_q value of 39.74 (Bhoora et al., 2009b; Chapter 3). The assay was able to detect *T. equi* parasite DNA in 59 of the 70 zebra samples with C_q values that ranged between 20.98 and 34.48, and in one sample, below the 95% sensitivity of the assay, at a C_q value of 39.14 (Table 4.2). The *T. equi* qPCR assay detected parasite DNA in all 17 *T. equi* RLB positive samples, as well as in 35 of the samples that tested negative on RLB and in the eight samples that hybridized to the *Theileria/Babesia* genus-specific probe only.

The detection limit (95% sensitivity) of the *B. caballi*-specific TaqMan MGB™ qPCR assay was shown to be 1.14 x 10^{-4}% parasiatemia at a C_q of 35.82 (Bhoora et al., 2009b; Chapter 3). For the *B. caballi*-specific qPCR assay, the upper limit of detection equated to a C_q value of 39.69 (Bhoora et al., 2009b). The *B. caballi* qPCR assay could detect *B. caballi* parasite DNA in 13 of the 70 zebra samples with C_q values that ranged between 29.85 and 35.74 (Table 4.2) and in 6 samples, below the 95% sensitivity of the assay, with C_q values between 36.08 and 39.40. Of these positive samples, *B. caballi* parasite DNA could be detected in seven samples that were positive for *T. equi* on RLB, four samples that hybridized to the *Theileria/Babesia* genus-specific probe only and in eight of the 45 negative RLB samples. All 19 *B. caballi* positive samples were also co-infected with *T. equi* as indicated by the *T. equi* qPCR assay.

Three overlapping nested PCRs resulted in the amplification of ~1480 bp of the complete 18S rRNA gene and nested PCR products were sequenced from 17 of the 70 zebra samples. Nine of the almost full-length 18S rRNA gene sequences were obtained from samples with single *T. equi* infections, and, although eight of the samples contained mixed *T. equi* and *B. caballi* infections, single 18S rRNA sequences were obtained from each of them. BLAST analysis revealed that all sequences obtained were most similar to previously published *T. equi* and not *B. caballi* 18S rRNA sequences. Phylogenetic analyses of the complete *T. equi* 18S rRNA gene sequences using the neighbor-joining, maximum-likelihood and Bayesian inference methods, yielded trees with almost identical topologies and high bootstrap or nodal support values. The phylogenetic analyses indicated the occurrence of the same three phylogenetic groups identified previously (Bhoora et al., 2009a; Chapter 2), designated A, B and C (Figure 4.1). While none of the complete 18S rRNA sequences from zebra grouped in clade A, twelve sequences grouped together with a *T. equi*-like isolate from a horse [strain ET1, accession number AY534882 (Nagore et al., 2004)] in clade B. Sequences grouping in clade B showed 98.6 – 98.9% identity to the *T. equi*-like strain ET1. Sequences representing this genotype were not detected previously in any South African horse samples (Bhoora et al., 2009a; Chapter 2), although similar sequences were identified in Cape mountain zebra from South Africa [*T. equi* RBEQ101, accession number EU642507; *T. equi* RBEQ96, unpublished (Bhoora et al., 2009a; Chapter 2)]. None of the new sequences from zebra were identical to EU642507, and five novel sequences were identified, with 99.5 – 99.8 % identity to EU642507. The remaining five *T. equi* 18S sequences occurred in clade C together with five previously published South African *T. equi* 18S sequences from horses [accession numbers EU888903, EU888905, EU642509, EU642510 and EU642511 (Bhoora et al., 2009a; Chapter 2)]. Three of the *T. equi* sequences from zebra (ZB29, ZB44 and ZB56) were identical to each other as well as to EU888903, while two novel sequences (ZB11 and ZB13), with 99.3 – 99.9% identity to EU888903, were identified (Figure 4.1).

Table 4.2 RLB and qPCR results obtained from zebra samples.

Sample ID	Species	RLB result	*B. caballi*-specific qPCR result	*T. equi*-specific qPCR result
ZB1	*E. quagga burchellii*	*Theileria/Babesia* catch all; *Babesia* catch all; *T. equi*	32.99	23.95
ZB2	*E. quagga burchellii*	Negative	Negative	26.25
ZB3	*E. quagga burchellii*	Negative	34.87	30.59
ZB4	*E. quagga burchellii*	*Theileria/Babesia* catch all; *Babesia* catch all	Negative	31.07
ZB5	*E. quagga burchellii*	Negative	34.09	24.35
ZB6	*E. quagga burchellii*	*Theileria/Babesia* catch all; *Babesia* catch all	Negative	30.87
ZB7	*E. quagga burchellii*	*Theileria/Babesia* catch all; *Babesia* catch all	36.13	27.81
ZB8	*E. quagga burchellii*	Negative	Negative	29.21
ZB9	*E. quagga burchellii*	*Theileria/Babesia* catch all; *Babesia* catch all	29.85	20.98
ZB10	*E. quagga burchellii*	*Theileria/Babesia* catch all; *Babesia* catch all	Negative	26.71
ZB11	*E. zebra zebra*	*Theileria/Babesia* catch all; *Babesia* catch all; *T. equi*	37.08	28.03
ZB12	*E. zebra zebra*	*Theileria/Babesia* catch all; *Babesia* catch all; *T. equi*	38.26	28.47
ZB13	*E. zebra zebra*	*Theileria/Babesia* catch all; *Babesia* catch all; *T. equi*	Negative	27.92
ZB14	*E. zebra zebra*	*Theileria/Babesia* catch all; *Babesia* catch all; *T. equi*	Negative	31.59
ZB15	*E. zebra zebra*	*Theileria/Babesia* catch all; *Babesia* catch all; *T. equi*	Negative	28.55
ZB16	*E. zebra zebra*	Negative	Negative	31.79
ZB17	*E. zebra zebra*	*Theileria/Babesia* catch all; *Babesia* catch all; *T. equi*	Negative	27.15
ZB18	*E. zebra zebra*	*Theileria/Babesia* catch all; *Babesia* catch all; *T. equi*	39.40	31.37
ZB20	*E. zebra zebra*	*Theileria/Babesia* catch all; *Babesia* catch all; *T. equi*	Negative	28.13
ZB21	*E. zebra zebra*	*Theileria/Babesia* catch all; *Babesia* catch all; *T. equi*	33.74	24.25
ZB22	*E. quagga burchellii*	Negative	Negative	26.86
ZB23	*E. quagga burchellii*	Negative	Negative	27.81
ZB24	*E. quagga burchellii*	*Theileria/Babesia* catch all; *Babesia* catch all; *T. equi*	35.47	22.37
ZB25	*E. quagga burchellii*	*Theileria/Babesia* catch all; *Babesia* catch all; *T. equi*	Negative	23.40
ZB26	*E. quagga burchellii*	*Theileria/Babesia* catch all; *Babesia* catch all; *T. equi*	34.45	24.77
ZB27	*E. quagga burchellii*	Negative	Negative	23.70
ZB28	*E. quagga burchellii*	*Theileria/Babesia* catch all; *T. equi*	Negative	30.78
ZB29	*E. quagga burchellii*	Negative	Negative	25.89
ZB30	*E. quagga burchellii*	Negative	Negative	28.85
ZB31	*E. quagga burchellii*	*Theileria/Babesia* catch all; *T. equi*	Negative	24.96

Sample ID	Species	RLB result	*B. caballi*-specific qPCR result	*T. equi*-specific qPCR result
ZB32	*E. quagga burchellii*	*Theileria/Babesia* catch all; *T. equi*	Negative	24.81
ZB33	*E. quagga burchellii*	*Theileria/Babesia* catch all; *T. equi*	Negative	32.19
ZB34	*E. quagga burchellii*	Negative	Negative	21.00
ZB35	*E. quagga burchellii*	Negative	Negative	26.91
ZB36	*E. quagga burchellii*	Negative	Negative	Negative
ZB37	*E. quagga burchellii*	Negative	Negative	Negative
ZB38	*E. quagga burchellii*	Negative	Negative	33.38
ZB39	*E. quagga burchellii*	Negative	Negative	30.38
ZB40	*E. quagga burchellii*	Negative	Negative	26.96
ZB41	*E. quagga burchellii*	Negative	34.15	21.71
ZB42	*E. quagga burchellii*	Negative	Negative	26.01
ZB43	*E. quagga burchellii*	Negative	Negative	Negative
ZB44	*E. quagga burchellii*	Negative	Negative	27.14
ZB45	*E. quagga burchellii*	Negative	32.14	25.77
EQ46	*E. quagga burchellii*	Negative	Negative	Negative
ZB47	*E. quagga burchellii*	Negative	Negative	29.13
ZB48	*E. quagga burchellii*	Negative	Negative	25.36
ZB49	*E. quagga burchellii*	Negative	Negative	25.57
ZB50	*E. quagga burchellii*	Negative	Negative	25.95
ZB51	*E. quagga burchellii*	Negative	Negative	30.38
ZB52	*E. quagga burchellii*	Negative	Negative	29.21
ZB53	*E. quagga burchellii*	Negative	Negative	32.54
ZB54	*E. quagga burchellii*	Negative	Negative	34.48
ZB55	*E. quagga burchellii*	Negative	35.74	28.95
ZB56	*E. quagga burchellii*	Negative	36.08	29.80
ZB57	*E. quagga burchellii*	Negative	32.92	26.52
ZB58	*E. quagga burchellii*	Negative	Negative	Negative
ZB59	*E. quagga burchellii*	Negative	Negative	39.14
ZB60	*E. quagga burchellii*	Negative	Negative	26.61
ZB61	*E. quagga burchellii*	*Theileria/Babesia* catch all; *Babesia* catch all	Negative	28.72
ZB62	*E. quagga burchellii*	Negative	Negative	Negative
ZB63	*E. quagga burchellii*	*Theileria/Babesia* catch all; *Babesia* catch all	37.31	24.51
ZB64	*E. quagga burchellii*	*Theileria/Babesia* catch all; *Babesia* catch all	34.97	23.84
ZB65	*E. quagga burchellii*	Negative	Negative	32.33
ZB66	*E. quagga burchellii*	Negative	Negative	Negative
ZB67	*E. quagga burchellii*	Negative	Negative	Negative
ZB68	*E. quagga burchellii*	Negative	Negative	30.61
ZB69	*E. quagga burchellii*	Negative	Negative	Negative
ZB70	*E. quagga burchellii*	Negative	35.22	23.33
ZB71	*E. quagga burchellii*	Negative	Negative	Negative

Figure 4.1 Rooted phylogram determined with Bayesian inference and 5 x 10^6 iterations using ~1480 bp of the full-length 18S rRNA sequences of *T. equi* samples identified in this study and *Theileria* and *Babesia* sequences from GenBank (accession numbers are indicated in parentheses). Posterior probabilities are indicated on the nodes of the tree. Where more than one identical sequence was obtained, one representative sequence is shown with the number of identical sequences obtained indicated in square brackets. ZB6 is representative of sequences obtained from ZB3, ZB30, ZB31, ZB32, ZB33, ZB34 and ZB64. Sequences obtained from ZB29, ZB44 and ZB56 are represented by *T. equi* LFEQ189 (EU888903).

Since it was not possible to directly sequence the full-length primary PCR product from all of the samples, the nested PCR product of the V4 hypervariable region (~670 bp) of the 18S rRNA gene was cloned and sequenced in order to obtain sequence data from more samples. In order to minimize the effects of PCR artifacts introduced during nested PCRs, a consensus sequence was obtained from six clones from each sample. A total of 33 18S rRNA V4 hypervariable region sequences were obtained from 31 samples. Seventeen sequences were obtained from 15 samples with mixed *T. equi* and *B. caballi* infections and 16 sequences from 16 samples with single *T. equi* infections. Once again, BLAST analysis revealed that all of the sequences were most similar to previously published *T. equi* 18S rRNA sequences. Phylogenetic analyses of the *T. equi* V4 hypervariable region sequences using neighbour-joining, maximum-likelihood and Bayesian inference methods again yielded trees with almost identical topologies and high bootstrap or nodal support values, and again, the same three phylogenetic groups were identified (Figure 4.2).

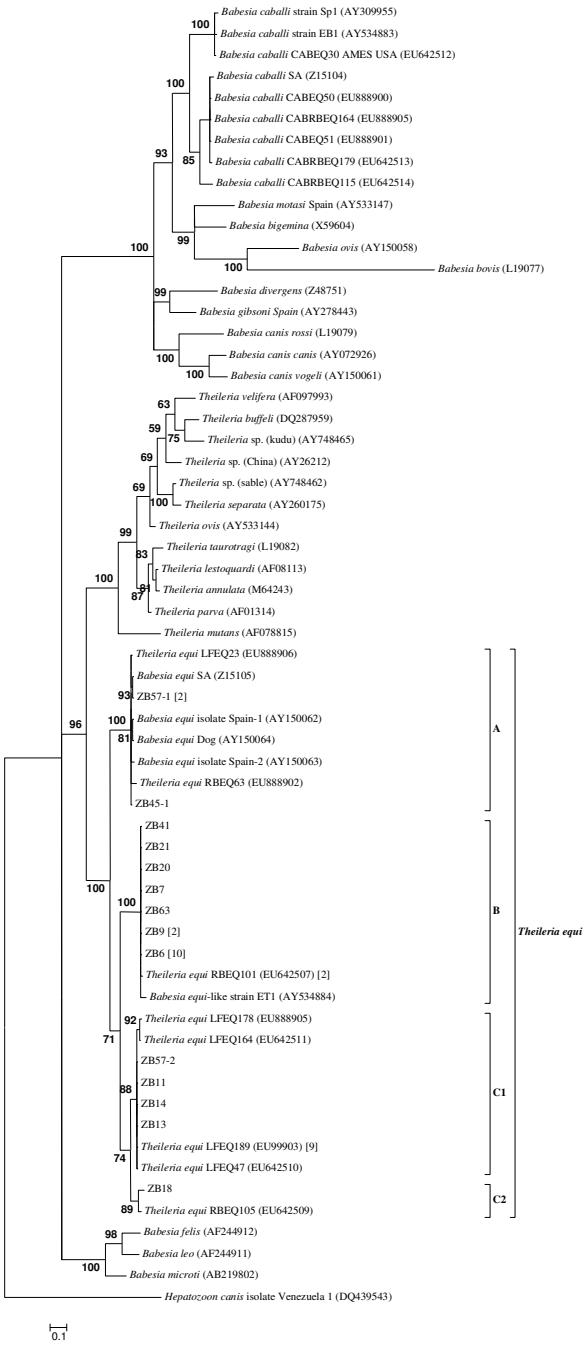

Figure 4.2 Rooted phylogram, determined with Bayesian inference and 5 x 10^6 iterations, of the V4 hypervariable region of the 18S rRNA gene sequences of *T. equi* samples identified in this study and *Theileria* and *Babesia* sequences from GenBank (accession numbers are indicated in parentheses). Posterior probabilities are indicated on the nodes of the tree. Where more than one identical sequence was obtained, one representative sequence is shown with the number of identical sequences obtained indicated in square brackets. One of the sequences obtained from sample ZB57 (ZB57-1) was identical to the published sequence *T. equi* RBEQ178 (EU642508). Identical sequences were obtained from ZB9 and ZB61. ZB6 is representative of sequences obtained from ZB1, ZB3, ZB10, ZB30, ZB31, ZB32, ZB33, ZB34 and ZB64. The sequence obtained from ZB24 was identical to *T. equi* RBEQ101 (EU642507). Similarly, *T. equi* LFEQ189 (EU888903) represents sequences obtained from ZB12, ZB15, ZB16, ZB29, ZB44, ZB45-2, ZB54 and ZB56.

Two zebra samples, ZB45 and ZB57, each contained *T. equi* parasites with two different V4 hypervariable 18S rRNA sequences; in each case the two different sequences grouped in different clades. Two of the *T. equi* 18S rRNA V4 hypervariable region sequences from zebra, ZB45-1 and ZB57-1, clustered within clade A, which contained previously published *T. equi* sequences from horses from South Africa [accession numbers: Z15105 (Allsopp et al., 1994), EU888906, EU642508, EU888902 (Bhoora et al., 2009a; Chapter 2)] and from Spain [accession numbers: AY150062; AY150063 and AY150064 (Criado-Fornelio et al., 2003)]. The ZB57-1 sequence was identical to two of the previously published South African *T. equi* 18S sequences, Z15105 and EU642508, while ZB45-1 showed 99.7% identity to these two sequences. Clade B contained 18 of the *T. equi* sequences from zebra, which showed 99.0% identity to *T. equi*-like strain ET1 [AY534882 (Nagore et al., 2004)], and between 99.5 and 100% identity to EU642507, previously identified in Cape mountain zebra from South Africa (Bhoora et al., 2009a; Chapter 2). Only one sequence (ZB24) was identical to EU642507. In addition to the five novel sequences identified by full-length 18S rRNA sequence analysis, two novel *T. equi* 18S rRNA V4 hypervariable sequences were identified. The remaining 13 *T. equi* sequences from zebra fell into the third phylogenetic group (clade C) which could be sub-divided into clades C1 and C2. Clade C1 contained 12 *T. equi* sequences from zebra, which grouped together with four recently published *T. equi* sequences from horses [accession numbers: EU888905, EU642510, EU642511 and EU888903 (Bhoora et al., 2009a; Chapter 2)]. Eight of the new sequences were identical to EU888903 while the remaining four sequences showed between 99.2 and 99.8% identity to the four published *T. equi* 18S rRNA sequences in clade C1. Clade C2 contained one new *T. equi* sequence from zebra, ZB18, which grouped together with a *T. equi* sequence from zebra that was characterized in a previous study [*T. equi* RBEQ105, accession number EU642509 (Bhoora et al., 2009a; Chapter 2)]. ZB18 showed 98.1% identity to EU642509.

4.5 Discussion

Molecular diagnostic assays developed for the detection of parasites that cause equine piroplasmosis are largely based on the 18S rRNA gene as a genetic marker (Bashiruddin et al., 1999; Battsetseg et al., 2001; Nicolaiewsky et al., 2001; Rampersad et al., 2003; Alhassan et al., 2007). Although nuclear ribosomal rRNA genes have been shown to provide appropriate targets to assist in the identification of species (Chae et al., 1998; Katzer et al., 1998; Allsopp and Allsopp, 2006), sequence heterogeneity has been reported within this gene in several species of

protozoa (Calder et al., 1996; Criado-Fornelio et al., 2003), including *T. equi* and *B. caballi* from horses in South Africa (Bhoora et al., 2009a; Chapter 2). Preliminary sequence data from three Cape mountain zebra samples further suggested that there may be more variation in *T. equi* genotypes in zebra in South Africa (Bhoora et al., 2009a; Chapter 2). In this study, we present sequence data from a larger group of zebra samples which substantiate our earlier findings.

RLB analysis indicated that 35% of the 70 zebra samples tested were positive for *Theileria* and/or *Babesia* piroplasm parasites. In contrast, the prevalence of piroplasm infection in South African horses as indicated by RLB analysis was shown previously to be approximately 63% based on a total of 488 samples collected from various geographical locations around the country (Bhoora et al., 2009a; Chapter 2). These results might suggest that equine piroplasmosis is not as widespread in zebra as it is in horses. However, further analysis using the recently developed TaqMan qPCR assay for *T. equi* (Kim et al., 2008) and the TaqMan MGB™ qPCR assay for *B. caballi* (Bhoora et al., 2009b; Chapter 3), enabled the detection of *T. equi* parasite DNA in 86% of samples and *B. caballi* in 27% of zebra samples tested. The high C_q values obtained by the qPCR assays for many of the samples indicate that the parasites are present at very low parasitaemia. We chose to use the RLB assay as it can detect novel genotypes and identify mixed infections; however, these advantages may be limited in equine piroplasmosis studies by the lack of sensitivity of the test. Both the RLB and the qPCR assays suggest that *T. equi* is more commonly found in zebra than *B. caballi*. This is similar to the observation in horses in South Africa, in which fewer cases of *B. caballi* infections are reported to occur (de Waal, 1990).

Although the RLB did not detect *Babesia* species-specific hybridization signals, nineteen samples were positive for *B. caballi* when tested using the *B. caballi*-specific TaqMan MGB™ qPCR assay. The C_q values reported were much higher than those obtained when samples were tested using the *T. equi*-specific qPCR assay, thus suggesting that the *B. caballi* parasitaemia was much lower than the *T. equi* parasitaemia in the zebra samples. This was further confirmed by cloning and sequencing of PCR products from mixed infections, which yielded only *T. equi* sequences. These results indicate that *B. caballi* infections in zebra may be similar to *B. caballi* infections in horses, in which the parasitaemia generally tends to be very low, rarely exceeding 1% (Hanafusa et al., 1998). It was therefore not possible to determine the level of intraspecific sequence variation in *B. caballi* genotypes that infect zebra in South Africa. In our previous study, however, we found that while there was less sequence variation amongst the *B. caballi* samples from horses, the *B. caballi* 18S rRNA gene sequences could be divided into two main

groups. We can therefore only speculate that there may also be less variation in the 18S rRNA gene sequences of *B. caballi* parasites infecting South African zebra than is observed for *T. equi* parasites of zebra.

Sequence analyses of both the full-length and the V4 hypervariable region of the *T. equi* 18S rRNA gene suggest the existence of 13 new *T. equi* and/or *T. equi*-like sequences from zebra. In many cases there were very few differences between the sequences, which could suggest that errors were introduced during nested PCRs. These differences are unlikely to be artifacts, however, as they were frequently present in sequences obtained from more than one sample. An additional sequence was identified in clade A, seven new sequences were identified in clade B and 5 in clade C. Thus, including the 12 *T. equi* 18S rRNA sequences that were previously identified in South African horse samples (Bhoora et al., 2009a; Chapter 2), a total of 25 *T. equi* 18S rRNA sequences have been identified, which belong to three main phylogenetic groups (Figure 4.1, Figure 4.2).

While the sequence differences in most cases were minimal, the results reported in this study indicate that there may be additional, as yet unidentified *T. equi* 18S rRNA sequences present in the horse and zebra populations in South Africa. The occurrence of previously unrecognized sequence variation could pose a potential problem in the implementation of the *T. equi* qPCR assay, as variation in the regions where the *T. equi* real-time primers and probe have been developed would render the assay non-specific. The *T. equi* TaqMan qPCR assay was, however, developed using a more conserved region of the 18S rRNA gene that occurs outside of the V4 hypervariable region, and this test has been able to detect all genotypes identified to date. Nonetheless, single nucleotide variations in the *T. equi* real-time primer regions and/or in the probe region were observed in two of the full-length sequences obtained in this study which grouped in clade B (ZB7 and ZB64), and in all sequences occurring in clade C, except *Theileria equi* RBEQ105 (accession number EU642509) (Figure 4.3).

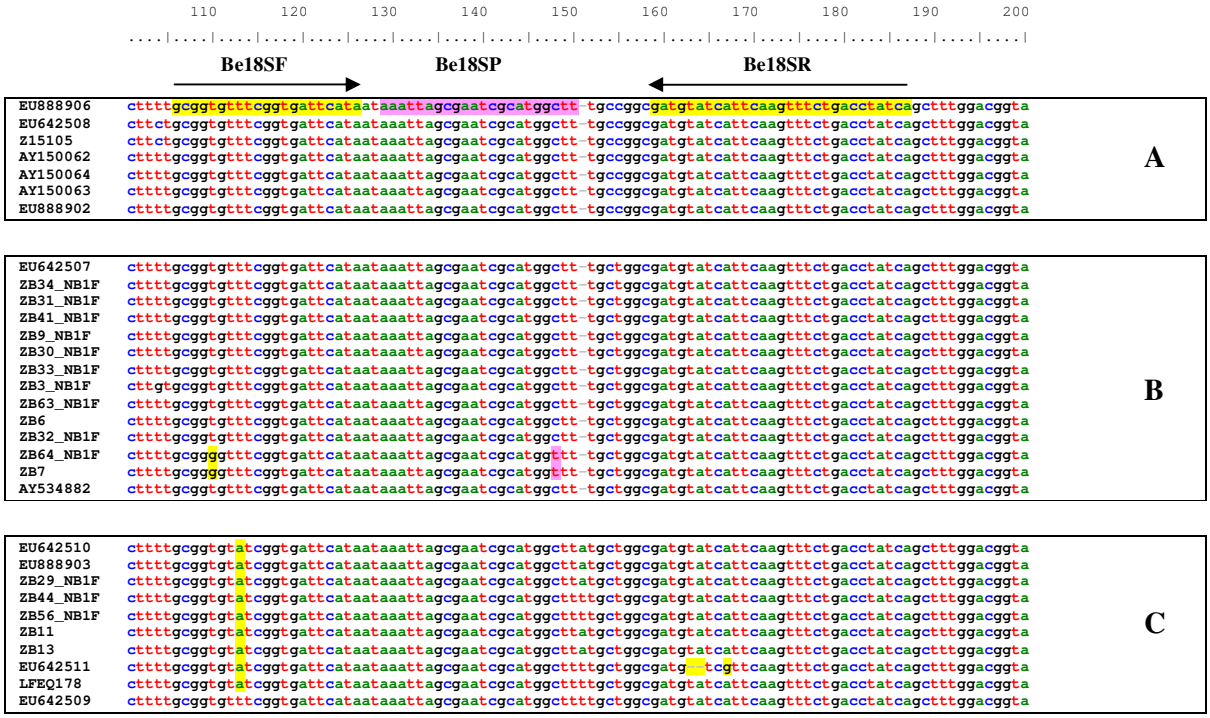

Figure 4.3 Nucleotide sequence alignment of a section of the 18S rRNA gene of the *T. equi* isolates examined in this study to published *T. equi* sequences (*B. equi* SA, Z151505; *B. equi* isolate Spain-1, AY150062; *B. equi* isolate Spain-2, AY150063; *B. equi* Dog, AY150064; *T. equi*-like strain ET1, AY534882; *T. equi* LFEQ23, EU888906; *T. equi* LFEQ178, EU888905; *T. equi* LFEQ189, EU88903; *T. equi* RBEQ63, EU888902; *T. equi* LFEQ164, EU642511; *T. equi* LFEQ47, EU642510; *T. equi* RBEQ105, EU642509; *T. equi* RBEQ178, EU642508; *T. equi* RBEQ101, EU642507). The *T. equi* real-time primers (Be18SF and Be18SR) and probe (Be18SP) sequences designed by Kim et al. (2008) are highlighted in yellow and pink, respectively. Nucleotide differences between sequences in the primer and probe regions are also highlighted in the same colours used to indicate the oligonucleotides. A, B and C represent the three *T. equi* 18S rRNA groups identified in this study.

Less than 10,000 years ago, horses became extinct in the Americas and the only survivors were horses in Asia (www.irishhorsesociety.com/horsedata/horseorigin.htm). Piroplasmosis of horses is therefore thought to be indigenous to Asia, but with the global movement of horses, both *T. equi* and *B. caballi* parasites have become distributed worldwide. When horses were introduced into Africa from Asia, they were almost certainly introduced along with their Asian piroplasm species. The diverse equid species indigenous to the African continent, including several species of zebra, probably carried closely related parasites, which evolved in Africa from a common ancestor of equid piroplasms and which could be transmitted by a wide range of tick vectors. Genetic recombination between the horse and zebra parasites, during sexual reproduction in the tick vectors, possibly led to the greater degree of genetic diversity of the 18S rRNA gene in equine parasites in this geographic region. As previously found for *T. equi* in horses, we have identified three groups of *T. equi* 18S rRNA sequences in zebra. Only two sequences from zebra were identified in group A, which contains sequences that are similar to

previously published *T. equi* sequences from horses in South Africa, Spain and the USA. The second group, group B, contains *T. equi*-like sequences identified in horses in Spain, but thus far only contains *T. equi*-like 18S rRNA sequences from zebra in South Africa. The third group contains new *T. equi* 18S sequences from both zebra and horses in South Africa. It appears that group A *T. equi* parasites occur more frequently in horses in South Africa while group B parasites appear to be more common in South African zebra, but unfortunately the number of zebra samples used in this study was not sufficient to infer an association between a specific parasite genotype and a particular zebra species. The results presented in this study therefore suggest that there exists an assortment of *T. equi* and *T. equi*-like 18S genotypes in all equid species. Examination of a larger sample population, containing samples from equal numbers of plains (*E. quagga*) and Cape mountain zebra (*E. zebra zebra*) species, may allow us to identify whether there is a prevalence of one particular genotype over the other in the different zebra species and might therefore indicate the original parasite for that equid species.

In conclusion, this study presents the first report on the molecular epidemiology of piroplasms infecting zebra in South Africa. The identification of 13 new *T. equi*-like 18S rRNA gene sequences confirms our earlier hypothesis that there is more variation in the *T. equi* genotypes in zebra in South Africa than in horses. Although we were able to confirm the presence of *B. caballi* infections in zebra using the recently developed *B. caballi*-specific MGB qPCR assay (Bhoora et al., 2009b; Chapter 3), sequence variation in the *B. caballi* 18S rRNA gene could not be determined due to the existence of extremely low circulating *B. caballi* parasitaemias in zebra.

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