

Sequence heterogeneity in the 18S rRNA gene within *Theileria equi* and *Babesia caballi* from horses in South Africa

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Abstract

A molecular epidemiological survey of the protozoal parasites that cause equine piroplasmiasis was conducted using samples collected from horses and zebra from different geographical locations in South Africa. A total of 488 samples were tested for the presence of *Theileria equi* and/or *Babesia caballi* using the reverse line blot hybridization assay. Ten percent of the samples hybridized to the *Theileria/Babesia* genus-specific probe and not to the *B. caballi* or *T. equi* species-specific probes, suggesting the presence of a novel species or genotype. The small subunit of rRNA gene (18S; ~1600 bp) was amplified and sequenced from 33 of these 488 samples. Sequences were compared with published sequences from the public sequence databases. Twelve distinct *T. equi* and six *B. caballi* 18S rRNA sequences were identified. Alignments demonstrated extensive sequence variation in the V4 hypervariable region of the 18S rRNA gene within *T. equi*. Sequence variation was also found in *B. caballi* 18S rRNA genes, although there was less variation than observed for *T. equi*. Phylogenetic analysis based on 18S rRNA gene sequences revealed three *T. equi* clades and two *B. caballi* clades in South Africa. The extent of sequence heterogeneity detected within *T. equi* and *B. caballi* 18S rRNA genes was unexpected since concerted evolution is thought to maintain homogeneity within repeated gene families, including rRNA genes, in eukaryotes. The findings reported here show that careful examination of variants of the 18S rRNA gene of *T. equi* and *B. caballi* is required prior to the development of molecular diagnostic tests to detect these parasites in horses. Species-specific probes must be designed in regions of the gene that are both conserved within and unique to each species.

Keywords: *Theileria equi*; *Babesia caballi*; Reverse line blot hybridization; 18S rRNA gene

1. Introduction

Equine piroplasmiasis is a tick-borne disease of equids that is caused by two species of apicomplexan protozoa, *Babesia caballi* and *Theileria equi* (Mehlhorn and Schein, 1998). The disease occurs throughout the tropical and subtropical areas of the world, with endemic areas in many parts of Europe, Asia, Arabia, South and Central America and Africa. *B. caballi* and *T. equi* are transmitted by species of ixodid ticks of the genera *Dermacentor*, *Rhipicephalus* and *Hyalomma* (de Waal and Van Heerden, 1994). Both species of protozoa cause infections, which can result in an acute or chronic disease, with mortalities of up to 50% (de Waal, 1992). The clinical signs of equine piroplasmiasis are often non-specific, complicating diagnosis. Therefore, it is not possible to differentiate between *T. equi* and *B. caballi* infections based on clinical signs alone (Potgieter et al., 1992) and mixed infections occur. Once infected, animals may remain life-long carriers of *T. equi* infections, while horses may remain carriers of *B. caballi* for up to 4 years (de Waal and Van Heerden, 1994).

Traditionally, the microscopic examination of blood smears is the method of choice for the detection and identification of blood parasites infecting horses (de Waal, 1992). However, due to the difficulties experienced in detecting low numbers of

parasites by microscopy in sub-clinically infected or carrier animals, serological assays, such as the complement fixation (CF) test, indirect fluorescence antibody test (IFAT), enzyme-linked immunosorbent assay (ELISA) as well as the competitive-inhibition ELISA (CI-ELISA) have been developed for the diagnosis of equine piroplasmosis (Knowles et al., 1991, Bruning et al., 1997, Kappmeyer et al., 1999 and Ikadai et al., 2000). However, there are limitations with serological assays due to problems with non-specificity, cross-reactivity and antibody detection limits (Bruning et al., 1997). Advances in molecular biological techniques have resulted in the improved detection, identification and genetic characterization of many haemoparasites (Caccio et al., 2000 and Nagore et al., 2004a). PCR has been applied for the detection of many species of *Babesia* and *Theileria* and has been shown to have higher sensitivity and specificity compared with serological assays (Geysen et al., 2003, Buling et al., 2007, Jefferies et al., 2007 and Sibeko et al., 2008). A sensitive and specific reverse line blot (RLB) hybridization assay, based on a sequence specific PCR that targets parasite 18S rRNA genes, has been developed for detection of *Theileria* and *Babesia* infections, including piroplasmosis in horses (Gubbels et al., 1999 and Nagore et al., 2004a). This assay enables the identification of mixed infections and has also proven to be a valuable tool in the identification of novel piroplasm species or genotypes (Georges et al., 2001, Criado-Fornelio et al., 2004, Nagore et al., 2004a, Nagore et al., 2004b and Nijhof et al., 2005). Nuclear ribosomal rRNA genes have been shown to provide appropriate targets to assist in the identification of species (Katzner et al., 1998, Chae et al., 1998 and Allsopp and Allsopp, 2006). However, sequence heterogeneity in the 18S rRNA gene has been reported within some species of protozoa (e.g. *Babesia bovis*, *Cytauxzoon felis*, *Theileria* sp. (type C)), both within a given isolate and among isolates from different geographical regions (Calder et al., 1996 and Criado-Fornelio et al., 2004). Therefore, although a high degree of 18S rRNA gene sequence conservation has been reported between *Babesia* and *Theileria* species, it has been recommended that the complete 18S rRNA gene of these parasites should be determined, particularly when dealing with new organisms, to ensure that genetic variation is not overlooked (Hunfeld et al., 2008). Although several PCRs, based on the 18S rRNA gene, have been developed for the detection of the parasites that cause equine piroplasmosis (Bashiruddin et al., 1999, Nicolaiewsky et al., 2001, Battsetseg et al., 2002, Rampersad et al., 2003 and Alhassan et al., 2007), the 18S rRNA gene sequence has been determined for only one isolate each of *T. equi* and *B. caballii* in South Africa. A preliminary study in our laboratory on the development of a real-time PCR assay for the specific detection of *T. equi* and *B. caballii* provided evidence of sequence heterogeneity in the V4 hypervariable region of the 18S rRNA gene sequences within each of these two species of piroplasm (unpublished findings). Therefore, the main purpose of this study was to explore levels of genetic heterogeneity within the *Theileria* and *Babesia* parasite species infecting horses in South Africa and make conclusions regarding the usefulness of diagnostic assays for equine piroplasmosis, which employ this V4 hypervariable region as a genetic marker.

2. Materials and methods

2.1. Field samples and tissue-culture stabulates

A total of 488 samples were investigated (Table 1). Blood samples, collected from 148 horses on six different stud farms in different locations in South Africa (Table 2), were obtained from the blood bank of the Equine Research Centre, Faculty of Veterinary Science, University of Pretoria. Also, 39 blood samples were collected from horses housed in the premises of this Faculty. Serum samples were collected at the South African National Two-year Sale (2005) and the National Yearling Sale (2006) and sent to the Agricultural Research Council-Onderstepoort Veterinary Institute where they were tested using IFAT. Based on these results, 211 horses, which tested positive serologically for *T. equi* and/or *B. caballii*, were identified and whole-blood samples were collected from each of them.

Table 1.

Origin and number of samples tested.

Origin	Number of samples
Stud farms ^a	148
Faculty of Veterinary Science, Onderstepoort	39
Field samples ^b	211
Tissue-culture samples	90
Total	488

^a For locations of the stud farms in South Africa see Table 2.

^b Field samples collected from horses at the South African National Two-year Sale (2005) and National Yearling Sale (2006).

Table 2.

Geographic location of stud farms in South Africa.

Farm	Place	Co-ordinates	Province	Number of samples
SA1	Port Elizabeth	S33.98 E25.51	Eastern Cape	18
SA2	Port Elizabeth	S34.02 E25.51	Eastern Cape	8
SA3	Port Elizabeth	S34.02 E25.50	Eastern Cape	3
SA4	Ceres	S33.36 E19.45	Western Cape	21
SA5	Colesberg	S30.65 E25.32	Northern Cape	64
SA6	Nottingham Road	S29.46 E29.91	KwaZulu Natal	32
Total				148

In addition, 90 tissue-culture samples, which included 17 Cape mountain zebra (*Equus zebra zebra*) samples from the Bontebok National Park, Western Cape province, South Africa (Zweygarth et al., 1997 and Zweygarth et al., 2002) were investigated.

2.2. DNA extraction

Genomic DNA was extracted from 200 µl of citrate-buffered or EDTA-treated blood or tissue-culture stabilate samples using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

2.3. 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 CTG 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 by Nijhof et al. (2005). The oligonucleotide probes used for the detection of *T. equi* and *B. caballi* are listed in Table 3.

Table 3.

Oligonucleotide probes used in the RLB hybridization assay.

Species	Probe sequence (5'–3')	Reference
<i>Theileria/Babesia</i> catch-all	TAATGGTTAATAGGARCRGTTG	Gubbels et al. (1999)
<i>Theileria</i> catch-all	ATTAGAGTGCTCAAAGCAGGC	Nijhof (unpublished)
<i>Theileria equi</i>	TTCGTTGACTGCGYTTGG	Butler et al. (2008)
<i>Babesia</i> catch-all 1	ATTAGAGTGTTC AAGCAGAC	Nijhof (unpublished)
<i>Babesia</i> catch-all 2	ACTAGAGTGTTC A AACAGGC	Nijhof (unpublished)
<i>Babesia caballi</i>	GTTGCGTTGTTCTTGCTTTT	Nijhof (unpublished)

Ambiguity codes: R = A/G; Y = C/T.

2.4. Sequencing and analyses

The complete 18S rRNA genes of parasites with novel genotypes were amplified using three different PCR primer sets. As it was not possible to amplify the 18S rRNA gene from samples with low piroplasm parasitaemia, the GenomiPhi DNA amplification kit (Amersham BioSciences) was used to amplify genomic DNA as described by the manufacturer, prior to performing nested PCR reactions.

Primers NBabesia1F and 18SRev-TB (Table 4) were used in a primary PCR to amplify a fragment of ~1600 bp. 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 genomic DNA. The cycling conditions were: an initial denaturation of 2 min at 94 °C, followed by 40 cycles of 30 s at 94 °C, 45 s at 60 °C and 1 min at 72 °C, and a final extension of 7 min at 72 °C. Three nested PCR reactions were subsequently performed. Primers NBabesia1F and BT18S3R (Table 4) were used to amplify an 800 bp PCR product at the 5'-end of the gene. The amplification program was the same as employed for the first PCR, except that an annealing step of 58 °C for 1 min was used. The amplification of the 3'-end of the 18S rRNA gene was accomplished using primers BT18S3F and 18SRev-TB (Table 4) with an annealing step of 55 °C for 1 min. An internal 800 bp product, which overlaps both the 5' and 3' fragments by ~400 bp, was amplified using primers BT18S2F and BT18S2R using an annealing step of 55 °C for 1 min.

Table 4.

Oligonucleotide primers used to amplify and sequence parasite 18S rRNA genes.

previously published *B. caballi* sequence from South Africa (accession number: Z15104) (Allsopp et al., 1994) were identified. Sequence alignments demonstrated extensive sequence variation in the V4 hypervariable region.

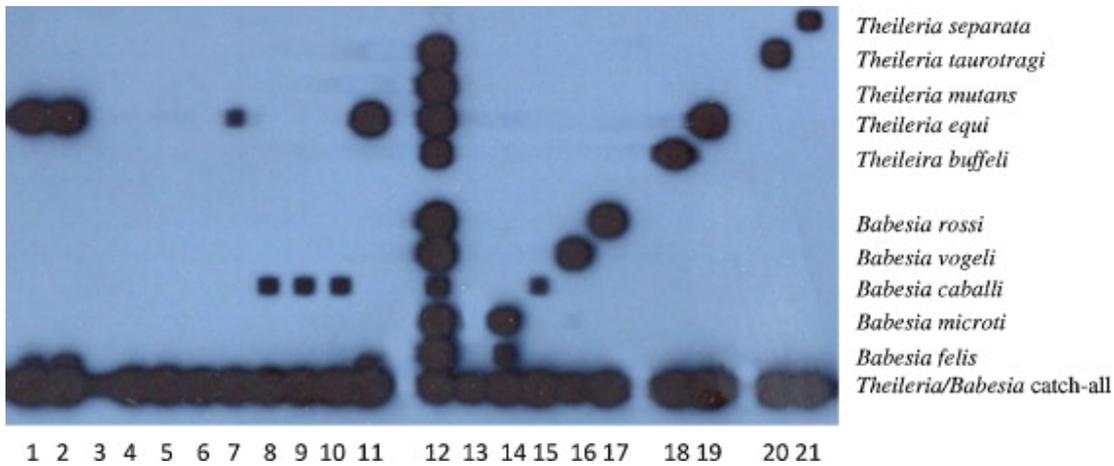


Fig. 1. Reverse line blot hybridization of *Theileria* and *Babesia* PCR products amplified from genomic DNA extracted from infected equine blood samples. Genus- and species-specific oligonucleotide probes were applied in horizontal rows as indicated. PCR products from samples and control clones were applied in vertical lanes. Lanes 1–11: PCR products from equine field samples. Lanes 12–21: PCR products from clones containing control DNA, specifically; lane 12: RLB plasmid control; lane 13: *Theileria/Babesia* genus-specific catch-all; lane 14: *B. felis* and *B. microti*; lane 15: *B. caballi*; lane 16: *B. vogeli*; lane 17: *B. rossi*; lane 18: *T. buffeli*; lane 19: *T. equi*; lane 20: *T. taurotragi*; lane 21: *T. separata*. Note that *T. equi* could be identified in samples 1, 2, 7 and 11, and *B. caballi* was present in samples 8–10, but samples 3–6 hybridized only to the *Theileria/Babesia* genus-specific probe.

Phylogenetic analyses using the neighbor-joining, maximum-likelihood and Bayesian inference, all yielded trees with almost identical topologies and high bootstrap or nodal support values. The analyses showed that the *T. equi* 18S rRNA gene sequences fell into three main groups, designated A, B and C (Fig. 2). Group A contained the previously published *T. equi* sequences from South Africa (accession number: Z15105) (Allsopp et al., 1994) and from Spain (accession numbers: AY150062, AY150063 and AY150064) (Criado-Fornelio et al., 2003). Group B contained two Cape Mountain zebra samples from the Western Cape Province of South Africa, which grouped together with a *T. equi*-like isolate from a horse (strain ET1, AY534882), reported previously (Nagore et al., 2004a). Sequences representing this genotype were not detected in any of the South African samples from horses. The third group (C) contained a number of new South African *T. equi* 18S sequences, which were distinct from all previously published 18S rRNA sequences from *T. equi*. *T. equi* samples from horses and/or zebras from different localities within South Africa were present in all three groups.

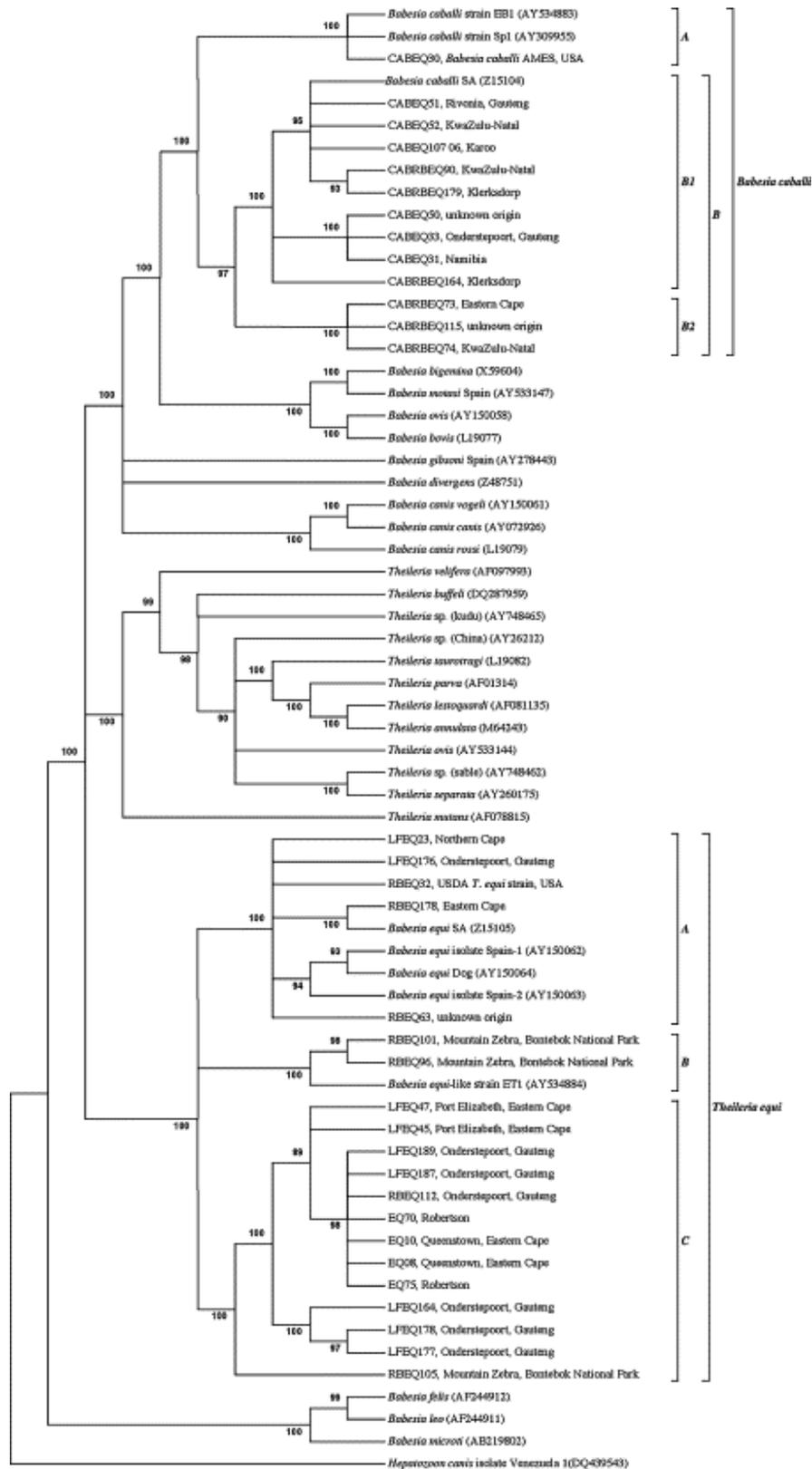


Fig. 2. Rooted cladogram, determined with Bayesian inference and 5×10^6 iterations, of the 18S rRNA gene sequences of *T. equi* and *B. caballi* samples identified in this study (square brackets) and *Theileria* and *Babesia* sequences from GenBank (accession numbers are indicated in parentheses). Posterior probabilities are indicated on the nodes of the tree.

While there was less sequence variation amongst *B. caballi* samples, the *B. caballi* 18S rRNA gene sequences could be divided into two groups (Fig. 2). Group A contained the published *B. caballi* sequences from Spain (AY309955 and AY534883) (Criado-Fornelio et al., 2004 and Nagore et al., 2004a) as well as a sequence obtained from a *B. caballi* USDA reference strain (*B. caballi* Ames, Iowa) (Kappmeyer et al., 1999). The South African samples fell into the second group (B), which could be sub-divided into sub-groups B1 and B2, the former sub-group containing the original South African *B. caballi* sequence (Z15104) (Allsopp et al., 1994).

The *T. equi* RLB probe used in this study could detect genotypes within group A, but not those within groups B and C. There were 15 nucleotide changes in the probe region between *T. equi* group A and B sequences, whereas there were 16 nucleotide differences for sequences within groups A and C (Fig. 3). The *B. caballi* RLB probe used in this study could detect most samples within group B, but not those within group A. The sequence of the RLB probe for *B. caballi* used in this study was the same in sequence to samples from group B1, but there was a point mutation in this region in group B2 (and in one sample, two point mutations) and six nucleotide differences in sequences in group A (Fig. 4).

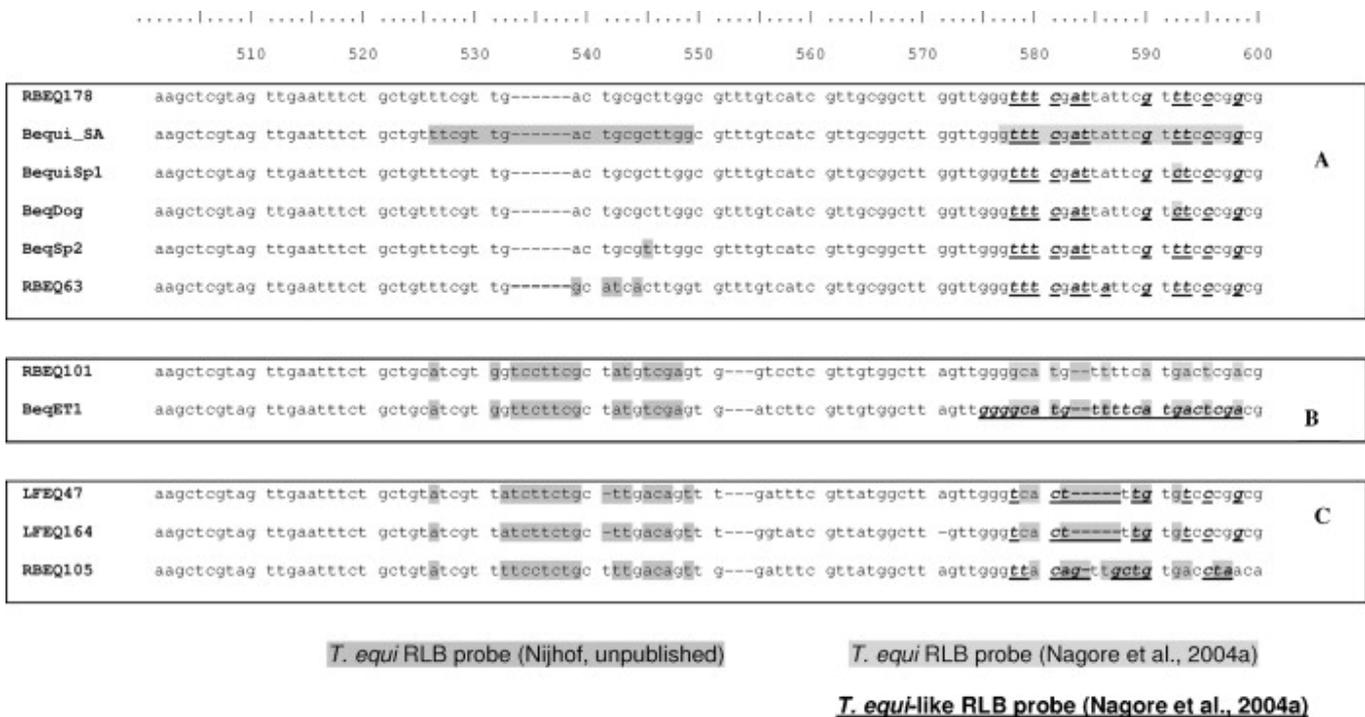


Fig. 3. Nucleotide alignment of a section of the V4 hypervariable region of the 18S rRNA gene of the *T. equi* isolates examined in this study, to the published *T. equi* sequences (*B. equi* SA, Z15105; *B. equi* Isolate Spain-1, AY150062; *B. equi* Isolate Spain-2, AY150063; *B. equi* Dog, AY150064; *T. equi*-like strain ET1, AY534882). The sequence of the *T. equi* RLB probe used in the present study is highlighted in grey. The sequences of the RLB probes designed by Nagore et al. (2004a) are either highlighted in blue or indicated in bold underlined italic font. Nucleotide differences between isolates in the probe regions are either highlighted in the same colors used to indicate the oligonucleotides, or represented in bold underlined italic font. (For interpretation of the references to color in the figure caption, the reader is referred to the web version of the article.)

Theileria and two other *Babesia* genotypes infecting Spanish horses (Criado-Fornelio et al., 2004 and Nagore et al., 2004a). Another study (Criado et al., 2006) showed genetically distinct *T. equi*-like isolates. Nagore et al. (2004a) designed RLB probes to detect both *T. equi* and *T. equi*-like 18S genotypes identified in their study. Their *T. equi* probe was designed to almost the same sequence region as our real-time PCR probe and was identical to most group A *T. equi* sequences (Fig. 3), but it was significantly different from sequences of members of group C identified in this study. The probe designed to detect *T. equi*-like parasites (Nagore et al., 2004a) would have been able to detect *T. equi* samples in group B, but in groups A and C there are twelve and thirteen nucleotide differences in the region of this probe. These differences would almost certainly prevent hybridization in the RLB assay, although this was not tested.

The nucleotide sequences of parasites from two Cape Mountain zebra samples (RBEQ96 and RBEQ101), from the Bontebok National Park in the Western Cape, were similar (99% identity) to the *T. equi*-like (*B. equi*-like strain ET1, AY534882) sequence (group B), but this genotype was not detected in any of the samples from horses in South Africa. A parasite 18S rRNA gene sequence from a third Cape Mountain zebra grouped in clade C, suggesting that there is more variation in *T. equi* genotypes in zebra in South Africa, but a study of a larger group of samples from zebra is required to substantiate these findings.

The RLB probe designed by Nagore et al. (2004a) to detect the *B. caballi* 18S genotype is five nucleotides longer at the 3'-end (Fig. 4), than the *B. caballi* RLB probe used in the present study. Sequence alignments indicated that both *B. caballi* RLB probes should have detected infections in most positive samples originating from South Africa, since these samples belonged to *B. caballi* groups B1 and B2 (although there are one or two nucleotide difference in the region of the RLB probe sequence within group B2 samples). However, in some cases, positive *B. caballi* samples were not detected in the RLB assay, but sequence analysis of some of these samples revealed *B. caballi* group B1 sequences. The inability of the RLB to detect all positive *B. caballi* infections is possibly due to very low parasitaemia in some samples, since it has been reported that the parasitaemia in *B. caballi* infections generally tends to be very low, rarely exceeding 1% (Hanafusa et al., 1998). The group A *B. caballi* sequences differed by six and eight nucleotides in the region of these two RLB probes (Fig. 4) suggesting that neither probe would have been able to detect samples in group A, which include *B. caballi* isolate Spain-1 (AY309955), *B. caballi*-like strain EB1 (AY534883) and the USDA isolate (CABEQ30, *B. caballi* AMES). On the other hand, the sequence of the *B. caballi*-like RLB probe also reported by Nagore et al. (2004a) is identical to the Spanish and American isolates, but differs from the South African group B1 and B2 *B. caballi* sequences at seven positions (Fig. 4).

Based on the sequence variation detected within each species of piroplasm, we determined whether primers and probes described by other authors would have amplified and detected the 18S rRNA gene from our newly identified South African *T. equi* and *B. caballi* genotypes. We were able to conclude that primers BEQF and BEQR, designed to amplify a 664 bp fragment from the 18S rRNA genome of *T. equi* (Bashiruddin et al., 1999), would not have been able to amplify all *T. equi* genotypes identified in this study. In addition, there were four nucleotide differences in the region where the *B. caballi* primer, BCAF, reported by the same authors, was designed. The reverse primer, BCAR, however, showed no nucleotide differences to other sequences in that region. These primers could therefore possibly amplify *B. caballi* DNA from all groups. This is consistent with the observation that sequence variation between the *B. caballi* samples investigated in the present study was not as extensive as that observed for *T. equi*. Rampersad et al. (2003) designed a nested PCR for the detection of *T. equi* based on the primers designed by Bashiruddin et al. (1999). Substantial sequence variation in groups B and C occurred in the region where their primers for the nested PCR (BEQF1 and BEQR1) were designed. More recently, a quantitative TaqMan assay, based on an 18S rRNA sequence outside of the V4 hypervariable region, has been developed for the detection of *T. equi* infections in horses (Kim et al., 2008). The 18S rRNA gene sequences

obtained in the present study were identical in the regions used to design the primers and probe for this *T. equi* TaqMan assay.

The extent of sequence heterogeneity detected here within *T. equi* and *B. caballi* 18S rRNA genes was unexpected. Multiple copies of rRNA genes are present in most eukaryotes and are thought to be highly homogenized owing to concerted evolution (reviewed by Eickbush and Eickbush, 2007). Although divergence from the concerted evolution model has been identified in *Plasmodium* species, which have functionally distinct rRNA 'types' (Gunderson et al., 1987), no evidence for functionally different rRNA genes has yet been found for *Theileria* or *Babesia* parasites (Dalrymple, 1990, Gardner et al., 2005 and Brayton et al., 2007). *Theileria parva* is recognised as a genetically and antigenically diverse parasite (Collins and Allsopp, 1999), and variants of this parasite can cause clinically quite distinct diseases in cattle (East Coast fever and Corridor disease). Despite the existence of such diversity in this parasite, a real-time PCR test specific for the detection of *T. parva* infections in buffalo has been developed using the 18S rRNA gene as a target (Sibeko et al., 2008) and to date, this test has not yet failed to detect *T. parva* (Sibeko, personal communication). While genetic variants within *T. equi* and *B. caballi* have been identified in Spanish horses based on 18S sequence data (Criado-Fornelio et al., 2004 and Nagore et al., 2004a), a recent study (Heim et al., 2007) did not reveal sequence variation in the 18S rRNA gene within either *T. equi* or *B. caballi* from horses in Brazil. In South Africa, the diverse equid species present are likely to carry closely related parasites that could be transmitted by a wide range of tick vectors. It is possible that genetic recombination during sexual reproduction in the tick vectors has led to the greater degree of sequence diversity of the 18S rRNA gene in equine parasites within this geographical region.

While the 18S rRNA gene sequences we have obtained in this study group most closely together with other *T. equi* and *B. caballi* sequences, some distinct and well-characterized species (e.g. *T. parva* and *T. annulata*) show higher levels of identity across their 18S rRNA sequences than the range for the two single equine species reported in this paper. We cannot rule out the possibility that the different groups that we have identified here represent different parasite species, although it is not possible to use 18S rRNA gene sequence variation alone to classify organisms as different species (Chae et al., 1999). However, all the field samples for which sequence data was obtained in this study were seropositive for either *T. equi* or *B. caballi*, although it is possible that this was due to previous infection or co-infection or cross-reactivity between closely related species.

The existence of sequence variation in the rRNA genes of the parasites that cause equine piroplasmiasis serves as a note of caution to researchers wishing to use rRNA genes for diagnostic purposes. Prior to the development of a molecular diagnostic test, it is recommended that a thorough survey should be carried out to assess levels of intraspecific sequence divergence in the genetic marker/s employed in the assay.

In conclusion, we have discovered extensive sequence variation in the 18S rRNA gene within *T. equi*, and have identified three distinct genetic groups of *T. equi* in South Africa. There was less sequence variation within *B. caballi* in this gene although fewer *B. caballi* samples were examined as there are fewer cases of *B. caballi* infections in South Africa (de Waal, 1990). The findings reported here show that careful examination of variants of the 18S rRNA gene of *T. equi* and *B. caballi* is required in order to identify regions that are both conserved within and unique to each species, before this gene can be considered as a target for the development of molecular diagnostic tests to detect these parasites in horses.

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