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Molecular genotyping of *Babesia caballi*

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ABSTRACT

Babesia caballi is an intra-erythrocytic parasite causing equine piroplasmosis. Three *B. caballi* genotypes (A, B, and C) have been identified based on the 18 S rRNA and rhoptry-associated protein (*rap*-1) gene sequences. These variant parasite genotypes compromise the diagnostic utility of the WOAH-recommended serological assays in declaring horses free of equine piroplasmosis. Although a gene encoding a spherical body protein 4 (*sbp*4) has recently been identified as a potential antigen for the serological detection of *B. caballi*, the ability of this antigen to detect the different geographical strains has not been determined. The molecular distinction between variant *B. caballi* genotypes is limited and therefore we developed molecular typing assays for the rapid detection and quantification of distinct parasite genotypes. Field samples were screened for the presence of *B. caballi* using an established multiplex equine piroplasmosis qPCR assay. In this study, *B. caballi* genotype A was not detected in any field samples screened. However, phylogenetic analysis of the amplified *sbp*4 and 18 S rRNA genes confirmed the phylogenetic groupings of the South African isolates into either *B. caballi* genotypes B or C. A multiple sequence alignment of the *sbp*4 gene sequences obtained in this study together with the published *sbp*4 sequences representing *B. caballi* genotype A, were used to identify conserved regions within the gene to design three primer pairs and three genotype-specific TaqMan minor-groove binder (MGB™) probes. The qPCR assays were shown to be specific and efficient in the detection and differentiation between *B. caballi* genotypes A, B, and C and could be used as a diagnostic assay to prevent the unintentional spread of variant *B. caballi* genotypes globally.

1. Introduction

The intraerythrocytic apicomplexan parasite, *Babesia caballi* is one of the causative agents of a globally significant disease of equids, referred to as equine piroplasmosis (EP) (Mehlhorn and Schein, 1998). Maintenance of the disease in its equid host species is reliant on the distribution of competent tick vectors (Schein, 1988). While EP is considered endemic in most tropical and sub-tropical parts of the world, it remains reportable to the World Organization for Animal Health (WOAH) as it negatively impacts the international movement of horses for trade and equestrian sporting events (WOAH, 2023). Although infections with *B. caballi* are considered less severe than *Theileria equi* infections, variable clinical manifestations of the disease make it difficult to distinguish between the two parasite infections based on clinical signs alone (de Waal, 1992).

Generally, the global prevalence of *B. caballi* has been reported to be much lower than that of *T. equi* (Bartolome Del Pino et al., 2023; Bashiruddin et al., 1999; Bhoora et al., 2009; Camino et al., 2021; Chen et al., 2022; Qablan et al., 2013; Rocafort-Ferrer et al., 2022). This can be attributed to the nature of *B. caballi* infections, which are typically self-limiting and can persist for up to four years without exhibiting any clinical signs until they are naturally cleared (de Waal, 1992; Friedhoff et al., 1990; Rothschild, 2013; Wise et al., 2013). Additionally, the sequestration of *B. caballi* parasites to the bone marrow results in parasitaemias that rarely exceed 1% in the blood of naturally infected horses (de Waal, 1992; Schein, 1988).

Despite this, phylogenetic analysis of the 18 S rRNA and *rap*-1 genes of *B. caballi* indicated the occurrence of three *B. caballi* clades denoted as A, B (or B1), and C (or B2) (Bhoora et al., 2009; Bhoora et al., 2010a; Rapoport et al., 2014). Recent investigations involving sequences from various countries, including Italy (Manna et al., 2018), Trinidad (Sant et al., 2019), Israel (Tirosh-Levy et al., 2020), and Paraguay (Ahedor et al., 2023), confirmed the clustering of *B. caballi* 18 S rRNA sequences into three distinct phylogenetic clades.

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South African *B. caballi* 18 S sequences clustered into clades B and C (Bhoora et al., 2009), together with isolates from Spain (Camino et al., 2020), Mongolia (Munkhjargal et al., 2013), Jordan (Qablan et al., 2013), Italy (Manna et al., 2018), Senegal (Dahmana et al., 2019) and Israel (Tirosh-Levy et al., 2020). Isolates from China (Wang et al., 2019), Spain (Criado-Fornelio et al., 2004), Brazil (Braga et al., 2017), Croatia (Beck et al., 2009), Paraguay (Ahedor et al., 2023) and Mongolia (Munkhjargal et al., 2013) clustered into clade A.

The *rap*-1 gene sequences of South African (Bhoora et al., 2010a) and Israeli (Rapoport et al., 2014) isolates consistently occurred within the same phylogenetic clades (B and C) observed for the 18 S rRNA gene, possibly indicating the presence of antigenically distinct *B. caballi* genotypes, that differ from those occurring in other parts of the world (Bhoora et al., 2009). Contradictory to these studies, investigations of the *rap*-1 gene sequences from samples in Mongolia (Munkhjargal et al., 2013), Cuba (Díaz-Sánchez et al., 2018) and Chile (Torres et al., 2021) identified four genotypes. A more recent study in China reported the clustering of *rap*-1 sequences into eight phylogenetic clades (Wu et al., 2023). Nevertheless, sequences from South Africa and Israel demonstrated clustering in clade 1, whereas sequences from Brazil, China, Cuba, Egypt, Indonesia, Mongolia, and Thailand were identified in the remaining seven clades (Wu et al., 2023).

As previously noted, the RAP-1 cELISA has been reported to exhibit poor performance in detecting *B. caballi* infections in isolates from South Africa (Bhoora et al., 2010a) and Israel (Rapoport et al., 2014). The existence of distinct genotypes and the failure of the WOAH-regulatory RAP-1 cELISA in detecting *B. caballi*-infected horses underscores the necessity for the development of alternative detection methods. This is crucial to prevent the accidental introduction or dissemination of novel genotypes into new areas (Bhoora et al., 2009, 2010a, 2010c).

The *B. caballi* SBP4 has recently been identified as a potential antigen that could be targeted in the development of novel serological assays (Mahmoud et al., 2020). The spherical body proteins play important roles in the interaction between the parasite and host, as well as the parasite's survival after erythrocytic invasion (Preiser et al., 2000; Terkawi et al., 2011b; Yokoyama et al., 2006). These proteins are released into the cytoplasm of red blood cells, making the environmental conditions favourable for parasite survival. This helps the parasites to grow and remain viable until infected cells rupture leading to the release of merozoites (Yokoyama et al., 2006).

The SBP4 antigen was first identified in *Babesia bovis* and proved to be an excellent target for serological tests because of its conservation between isolates from Africa, America, and Asia (Terkawi et al., 2011a). Furthermore, these proteins are highly immunogenic as they are released into the cytoplasm of infected erythrocytes during the later stages of infection (Terkawi et al., 2011b). Recombinant *B. bovis* SBP4 antigens used in the development of an indirect ELISA (iELISA), showed high sensitivity and specificity in the detection of *B. bovis* infections in carrier cattle (Chung et al., 2017; Terkawi et al., 2011a).

Similarly, an iELISA using a recombinant *B. caballi* SBP4 antigen was able to detect all *B. caballi* infections that were confirmed positive with PCR and indirect immunofluorescent antibody tests (IFAT) (Chung et al., 2017; Mahmoud et al., 2020). The results of the *Bc*SBP4-iELISA correlated with the IFAT, which was considered the reference test in the study performed by Mahmoud et al. (2020). Although the *Bc*SBP4-iELISA showed promising results in the detection of *B. caballi* infections in samples from America and Egypt, additional research is needed to ascertain the conservation of the SBP4 antigen in *B. caballi* samples globally (Mahmoud et al., 2020).

Molecular typing is an alternative approach that can be considered to improve the diagnosis of EP. The successful development of the *T. equi* molecular typing multiplex-qPCR assays enabled the detection and differentiation between the five variant *T. equi* genotypes (Bhoora et al., 2020). The *T. equi* genotyping assays facilitated the assessment of intra-and interspecies variation in field samples, hypothesising that the higher genotypic variation in zebra indicates that they could be the ancestral hosts of these parasites. Similarly, the utility of *B. caballi* molecular typing qPCR assays becomes significant, particularly when considering the implications of transporting horses infected with novel parasite genotypes across borders. In the present study, we describe the development of TaqMan Minor Groove Binder™ (MGB™) qPCR assays for the detection and differentiation between the three *B. caballi* genotypes. When used in conjunction with the RAP-1 cELISA or the *Bc*SBP4-iELISA, these typing qPCR assays have the potential to significantly improve the accuracy of detecting *B. caballi* infections globally.

2. Materials and methods

2.1. Samples

Retrospective DNA samples isolated from South African field horses (*Equus caballus*) (n=170) and zebra (*Equus quagga burchelli*, *Equus zebra hartmannae,* and *Equus zebra*) (n=157) (Table 1) were included in this study. Additionally, *in vitro* cultured isolates (n=5) established and maintained in our laboratory and *Theileria haneyi* positive control DNA obtained from a previous study conducted in our laboratory (Katjivena, 2022), were also included in our analysis. DNA was extracted from *in vitro* cultured isolates using the PureLink® Genomic DNA Mini Kit (Thermo Fisher Scientific™, South Africa) according to the manufacturer's instructions.

2.2. Screening samples for the presence of T. equi and B. caballi

DNA samples were screened for the presence of equine piroplasmosis using the Multiplex-EP qPCR assay (Bhoora et al., 2010c; Bhoora et al., 2018; Kim et al., 2008). The assay was adapted for use on the QuantStudio™ 5 Real-Time PCR system (Applied Biosystems) by modifying the fluorescent dyes of the probes (Table 2). Real-time PCRs were performed using MicroAmp optical 96-well reaction plates as previously described (Bhoora et al., 2018). Data was analysed using the QuantStudio™ Design and Analysis software v2.6.0. (Applied Biosystems).

2.3. Characterization of the B. caballi sbp4 and 18 S rRNA genes

2.3.1. Amplification of the B. caballi sbp4 gene

Preliminary whole genome sequence data, available for South African *B. caballi* isolates representing genotypes B and C (unpublished data), was used to design two sets of primer pairs to amplify the *sbp*4 gene from South African field isolates. Primers were designed using

Table 1
Origin and number of retrospective DNA samples from South African field horses and zebras.

Origin	Animal species	Province	Number
Equine Clinic Onderstepoort	*E. caballus*	Gauteng	5
Equine Research Centre	*E. caballus*	Gauteng	2
Olympus	*E. caballus*	Gauteng	11
Ntembeni	*E. caballus*	KwaZulu-Natal	31
Summerveld	*E. caballus*	KwaZulu-Natal	6
Tafuleni	*E. caballus*	KwaZulu-Natal	49
Vryheid	*E. caballus*	KwaZulu-Natal	30
Louwna	*E. caballus*	North-West	36
Kruger National Park	*E. quagga burchellii*	Limpopo and Mpumalanga	57
Mountain Zebra National Park	*E. zebra zebra*	Eastern Cape	20
Augrabies Falls National Park	*E. zebra hartmannae*	Northern Cape	20
Mokala National Park	*E. quagga burchellii*	Northern Cape	20
Bontebok National Park	*E. zebra zebra*	Western Cape	20
Karoo National Park	*E. zebra zebra*	Western Cape	20
Total			327

Table 2
Sequences of primer sets and probes used in the Multiple-EP qPCR assay.

Species	Primer/Probe	Sequence 5'-3'	References
Theileria equi	Be_18SF	GCGGTGTTTCGGTGATTCATA	Kim et al., 2008
	Be_18SR	TGATAGGTCAGAAACTTGAATGATACATC	
	Be_18SP	6-FAM-AAATTAGCGAATCGCATGGCTT-QSY	
Babesia caballi	Bc_18SF402	GTAATTGGAATGATGGCGACTTAA	Bhoora et al., 2010c
	Bc_18SR496	CGCTATTGGAGCTGGAATTACC	
	Bc_18SP	VIC-CCTCGCCAGAGTAA-MGB	

Primer Express 3.0.1 (Applied Biosystems) and synthesised by Inqaba Biotechnical Industries (Pty) Ltd (South Africa). Primers *Bcsbp*4-B_FWD (5'-CACGTACGAGGCCAACTA-3') and *Bcsbp*4-B_REV (5'-TCGACGAGGTGGTAGAAGA-3') were used to amplify a 656 bp fragment of the *sbp*4 gene from *B. caballi* genotype B, while primers *Bcsbp*4-C_FWD (5'-CACCTTCGAGGGCAACTAC-3') and *Bcsbp*4-C_REV (5'-AAATCTCCTCGTAGGGACCA-3') were used to amplify a 636 bp fragment of the *sbp*4 gene from *B. caballi* genotype C. The *B. caballi sbp*4 genes representing genotypes A, B, and C are hereafter referred to as *Bcsbp*4-A, *Bcsbp*4-B, and *Bcsbp*4-C, respectively.

PCRs were performed in triplicate, in 0.2 ml thin-walled PCR tubes using the MiniAmp™ Thermal Cycler (Applied Biosystems). The reactions consisted of 12.5 µl DreamTaq Green PCR Master Mix (Thermo Fisher Scientific™, South Africa), 0.2 µM of each primer, 5 µl genomic DNA, and 6.5 µl water in a final volume of 25 µl. Cycling conditions included an initial denaturation for 3 minutes at 95°C, followed by 35 cycles of 30 seconds at 95°C, 40 seconds at 54°C, and 1 minute at 72°C and the final extension of 7 minutes at 72 °C.

2.3.2. Amplification of the V4 hypervariable region of the 18 S rRNA gene

The V4 hypervariable region of the 18 S rRNA gene was amplified from seven *B. caballi sbp*4-positive samples containing either genotype B or C infections. *Theileria/Babesia* genus-specific primers, RLB-F2 (5'-GACACAGGGAGGTAGTGACAAG-3') and biotin-labelled RLB-R2 (5'-Biotin-CTAAGAATTTCACCTCTAACAGT-3'), were used in the amplification of the 430 bp V4 hypervariable region (Nijhof et al., 2003; Nijhof et al., 2005).

PCRs were performed in triplicate for each sample in 0.2 ml thin-walled PCR tubes using the Veriti™ 96-Well Fast Thermal Cycler (Applied Biosystems). Reactions consisted of 12.5 µl Phusion Flash High-Fidelity PCR Master Mix (Thermo Fisher Scientific™, South Africa), 0.2 µM of each primer, 2.5 µl genomic DNA, and 9.5 µl water in a final volume of 25 µl. Cycling conditions included an initial denaturation for 10 seconds at 98°C, followed by 35 cycles of 1 second at 98°C, 5 seconds at 58°C and 15 seconds at 72°C, and the final extension of 1 minute at 72°C. All amplicons were visualised by 1.5% (w/v) agarose gel electrophoresis.

2.3.3. Cloning, sequencing, and phylogenetic analysis

Respective replicate amplicons generated from the *sbp*4 and 18 S rRNA PCRs were pooled and purified using the PureLink™ Quick Gel Extraction and PCR Purification Combo Kit (Invitrogen™). The *sbp*4 and 18 S rRNA gene amplicons were respectively cloned into the pJET1.2/blunt cloning vector using the CloneJET PCR Cloning Kit (Thermo Fisher Scientific™, South Africa). Plasmid DNA isolation was performed with the GeneJET™ Plasmid Miniprep Kit (Thermo Fisher Scientific™, South Africa).

Plasmid DNA samples from *sbp*4 (n=13) and 18 S rRNA (n=19) recombinant clones were sent for Sanger sequencing to the Central Analytical Facilities at the University of Stellenbosch, South Africa. The plasmid inserts were sequenced using the pJET1.2 forward sequencing primer and the pJET1.2 reverse sequencing primers (Thermo Fisher Scientific™, South Africa).

The quality of the sequences was evaluated using Chromas 2.6.6 Software (Technelysium Pty Ltd). The sequences were assembled and edited using GAP4 of the Staden Package software suite (Staden, 1996) and BLASTn was used to search the public sequence databases for homologous nucleotide sequences (Altschul et al., 1990). BLASTP was used to compare the amino acid identities of the proteins translated from the *sbp*4 genes (Altschul et al., 1990). The sequences were deposited in GenBank under accession numbers: PP459003, PP459004, PP459005, PP459006, PP459007, PP459008, PP459009.

The nucleotide consensus sequences of each gene (*sbp*4 and 18 S rRNA) were aligned, respectively, using ClustalW multiple alignment software (Thompson et al., 1994) and all manual adjustments of the alignments were conducted using BioEdit (version 7.2) (Hall, 1999).

Phylogenetic analysis of the *sbp*4 gene and the V4 hypervariable region of the 18 S rRNA gene sequences was carried out using the maximum-likelihood and neighbour-joining methods in MEGA11 (Tamura et al., 2021). The appropriate models for the analysis of each gene were determined using MLModelTest (MEGA11). Consequently, the Tamura 3-parameter substitution model with a proportion of invariable sites (T92 + I) was selected for the *sbp*4 gene and the Jukes-Cantor (JC) substitution model with uniform rates for the 18 S rRNA gene. The maximum-likelihood and neighbour-joining phylogenetic trees were constructed with 1000 bootstrap replicates using MEGA11 (Tamura et al., 2021). The *Babesia bigemina sbp*4 gene sequence (XM012912519) (Jackson et al., 2014) was included as an outgroup in the *sbp*4 phylogenetic tree, while the *Babesia divergens* 18 S rRNA gene sequence (Z48751) (Skuce et al., 1996) was included as an outgroup in the analysis of the sequences of the V4 hypervariable region of the 18 S rRNA gene.

2.4. Development of B. caballi typing qPCR assays

The multiple sequence alignment of the *sbp*4 gene against the published *B. caballi sbp*4 reference sequences (MT032179.1 and MT032180.1) (Mahmoud et al., 2020) enabled the identification of conserved regions for the design of genotype-specific qPCR assays (Fig. 1).

Primer pairs (Integrated DNA Technologies) and TaqMan MGB™ probes (Applied Biosystems), targeting the *Bcsbp*4-A, *Bcsbp*4-B, and *Bcsbp*4-C genes respectively, were designed using Primer Express 3.0.1 (Applied Biosystems) (Table 3). The primers and probes were evaluated for specificity *in silico* using BLASTn (Altschul et al., 1990).

The *B. caballi* genotyping qPCR assays (*Bcsbp*4-A, *Bcsbp*4-B, and *Bcsbp*4-C) were performed using the QuantStudio™ 5 Real-Time PCR system (Applied Biosystems). The reactions consisted of 10 µl KAPA Probe Universal Mix (KAPA biosystems), 0.9 µM of the genotype-specific forward and reverse primer, 0.25 µM of the TaqMan MGB™ genotype-specific probe, and 2.5 µl of the target DNA in a final volume of 20 µl. The cycling conditions included enzyme activation at 95°C for 20 seconds, followed by 40 cycles of 1 second at 95°C and 20 seconds at 60°C. Amplification data was analysed using the QuantStudio™ Design and Analysis software v2.6.0. (Applied Biosystems).

2.4.1. Sensitivity, specificity, and efficiency of the B. caballi typing qPCR assays

Custom *B. caballi* genotype-specific plasmid controls were synthesized by Invitrogen Gene Art Synthesis (Thermo Fisher Scientific™,

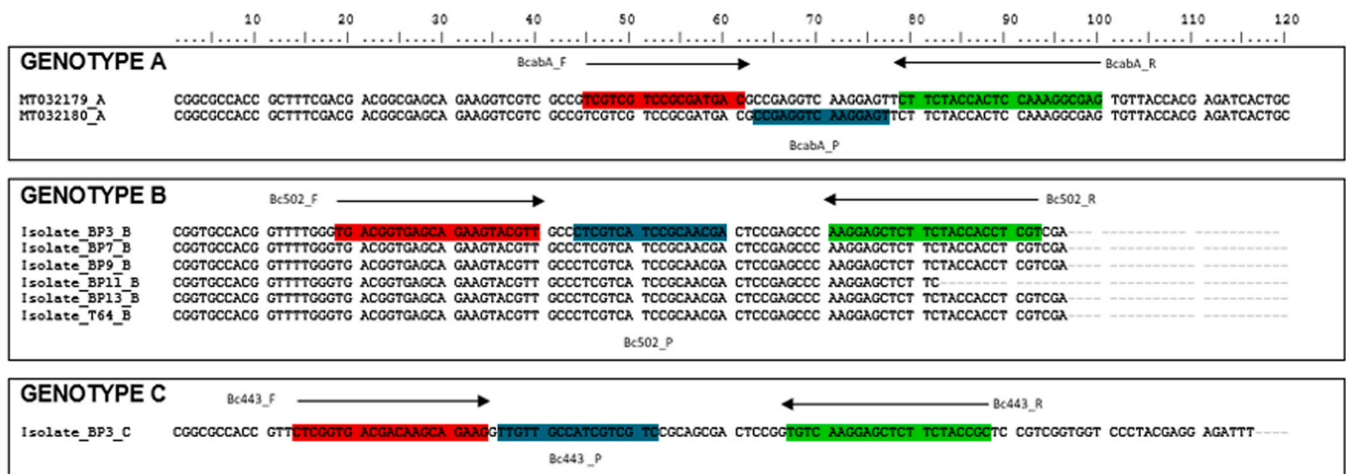

Fig. 1. Nucleotide sequence alignment of a section of the *B. caballi sbp*4 gene obtained in this study to published *B. caballi sbp*4 reference sequences, MT032179 and MT032280. The sequences of the genotype-specific forward primers are highlighted in red, the reverse primers in green and the probes are highlighted in blue.

Table 3
Sequences of *Babesia caballi* genotype-specific primer sets and probes designed for *B. caballi* typing qPCR assays.

Babesia caballi genotype	Primer/Probe ID	Sequence 5'-3'
A	BC_SBP4A_F	TCGTCGTCCGCGATGAC
	BC_SBP4A_R	TCGCCTTTGGAGTGGTAGAAG
	BC_SBP4A_P	NED-CCGAGGTCAAGGAGT-MGB
B	BC_SBP4B_F	TGACGGTGAGCAGAAGTACGTT
	BC_SBP4B_R	ACGAGGTGGTAGAAGAGCTCCTT
	BC_SBP4B_P	6-FAM-CTCGTCATCCGCAACGA-MGB
C	BC_SBP4C_F	CTCGGTGACGACAAGCAGAAG
	BC_SBP4C_R	GCGGTAGAAGAGCTCCTTGACA
	BC_SBP4C_P	VIC-TTGTTGCCATCGTCGTC-MGB

South Africa). The concentration of the plasmid constructs was determined using the Xpose spectrophotometer (Unchained Labs, Germany). The copy number of each construct was calculated, and the concentration was adjusted to 10^9 copies/µl for genotypes A and B, and to 10^7 copies/µl for genotype C. A ten-fold serial dilution for each genotype-specific plasmid construct was prepared. The dilution series ranged from 10^9 to 1 copies/µl for genotype A and B plasmid constructs, and 10^7 to 1 copies/µl for genotype C.

For each plasmid standard dilution, the genomic equivalent per µl was converted into % parasitaemia (% "parasitized" erythrocytes; PE) by assuming an average of 9 $\times 10^6$ equine red blood cells/µl (Latimer and Duncan, 2011).

Amplification of the ten-fold dilution series of each of the three plasmid controls was performed in triplicate and on five separate occasions. The data generated from the replicate runs for each plasmid control, were used to calculate the linear regression equations of the cycle threshold (C_T) values plotted against the calculated log % parasitaemia, from which the efficiency of each of the assays could be determined. To determine the limit of detection (LOD), probit analysis was conducted over the non-linear range of each respective assay. For each plasmid standard, five replicates of a 2-fold dilution series were tested over three consecutive days.

The analytical specificity of each assay was determined by testing DNA from *T. equi* and *T. haneyi*, which are protozoal parasites known to infect equids. Cross-reactivity between the probes was also assessed by testing the probes against each of the *B. caballi* genotype-specific plasmid controls.

The diagnostic efficiency of each of the developed *B. caballi* molecular typing assays was determined by screening previously confirmed *B. caballi* positive field samples (n=18) and *in vitro* cultured isolates (n=5). The Kappa coefficient was used to determine the level of agreement between the conventional *sbp*4 PCR and the molecular typing qPCR assays developed for genotypes B and C.

3. Results

3.1. Molecular detection of equine piroplasmosis

Retrospective DNA samples (n=327) were screened for *T. equi* and *B. caballi* parasites using the Multiplex-EP qPCR assay (Bhoora et al., 2010c; Bhoora et al., 2018; Kim et al., 2008). Single *T. equi* infections were detected in 285 (87%) samples (138 from horses, 147 from zebras) while mixed infections with both parasites were detected in 14 (4%) horse samples and single *B. caballi* infections in four (1%) samples (3 from horses, and 1 from a zebra). Neither *T. equi* nor *B. caballi* could be detected in 24 (7%) samples (n=15 horses, n=9 zebras). Of the five *in vitro* cultured isolates, only one isolate (BP11) displayed a mixed infection with both parasites, while the remaining four were positive for *B. caballi* only.

3.2. Characterization of the B. caballi sbp4 gene

The *sbp*4-based conventional PCR assays designed for the detection of *B. caballi* genotypes B and C were used to screen *B. caballi*-positive field samples (n=18) and *in vitro* cultured isolates (n=5). Five (T25, T64, T73, T75, and T158) of the 18 field samples tested positive for *B. caballi* genotype B with an expected amplicon size of 656 bp, while none tested positive for genotype C. Mixed infections with *B. caballi* genotypes B and C (636 bp amplicon) were however detected in three *in vitro* cultured isolates (BP3, BP9, and BP11), while single genotype B infections were identified in two isolates (BP7 and BP13).

Amplicons from the ten *B. caballi sbp*4-positive samples were selected for cloning and sequencing. Twenty colonies, representing two colonies from each sample, were screened to confirm recombinants. Colony PCR analysis using the *sbp*4-specific primers confirmed the presence of the *sbp*4 gene in 13 of the colonies screened. Twelve clones represented *B. caballi* genotype B and one *B. caballi* genotype C.

A total of eight *sbp*4 sequences, representing the five *B. caballi in vitro* cultured isolates (BP3, BP7, BP9, BP11, BP13) and two field samples (T64, and T73), were obtained from the 13 recombinant clones. Seven sequences represented genotype B, and one genotype C. Two of these sequences were obtained from one *in vitro* cultured isolate (BP3), containing a mixed infection; with one sequence representing genotype B and the other genotype C.

The maximum-likelihood and neighbour-joining phylogenetic analyses of the *sbp*4 gene sequences were performed to evaluate the evolutionary relationships of the South African *B. caballi* isolates to published sequences. Phylogenetic trees with identical topologies and high bootstrap values were obtained from the two analyses, confirming three *B. caballi* groups previously designated as A, B, and C (Bhoora et al., 2009; Bhoora et al., 2010a). Published *sbp*4 sequences from the USA and Egypt grouped into clade A (MT032179 and MT032180) (Mahmoud et al., 2020), while the *sbp*4 sequences obtained from seven South African isolates (BP3B, BP7, BP9, BP11, BP13, T64, and T73) (Accession numbers: PP459003, PP459004, PP459005, PP459006, PP459007, PP459008) clustered within clade B, and only one sequence (BP3C) (Accession number: PP459009) occurred in clade C (Fig. 2). BLASTn analysis revealed that all the *B. caballi sbp*4 gene sequences obtained in this study shared 82.8–83.2% identity with reference sequences (MT032179 and MT032180) in clade A. Similarly, BLASTP analysis of the translated proteins of the *sbp*4 gene sequences obtained during this study shared 68.5–70.7% identity with the translated reference protein sequences (MT032179 and MT032180). Four sequences in clade B (BP3B, BP7, BP9 and T64) were identical to each other, while the remaining three sequences (BP11, BP13 and T73) showed 99–99.8% identity to each other. The *sbp*4 gene sequence in clade C (BP3C) showed between 84.9% and 85.3% identity to sequences occurring in clade B.

3.3. Characterization of the B. caballi 18 S rRNA gene

The V4 hypervariable region of the 18 S rRNA gene (~430 bp) was amplified from seven samples from which *sbp*4 gene sequences were obtained. The amplicons were cloned, and colony PCR analysis of three colonies per sample confirmed the presence of the V4 hypervariable region in 19 colonies.

Nine sequences, representing the seven samples were obtained from the 19 recombinant clones. BLASTn analysis revealed that two sequences (BP11 and T73) showed 99.3–100% identity to *T. equi* sequences belonging to genotype A (JX177673) (Hall et al., 2013). The two *T. equi* sequences were excluded from further analysis as the focus of this study was on *B. caballi*. An additional sequence obtained from the BP11 recombinant clone identified as *B. caballi*, indicated a mixed infection with both parasites. In total, seven *B. caballi* 18 S rRNA gene sequences were obtained from five *in vitro* cultured isolates (BP3, BP7, BP9, BP11, and BP13) and one field sample (T64). One *in vitro* cultured isolate, BP3, had two sequences (BP3B and BP3C) representing genotypes B and C, respectively.

The maximum-likelihood and neighbour-joining phylogenetic analyses of the sequences of the V4 hypervariable region of the 18 S rRNA gene were performed to confirm the evolutionary relationship of the South African *B. caballi* sequences obtained in this study, with published sequences. Phylogenetic trees with identical topologies and high bootstrap values were obtained, confirming the groupings of the sequences into three 18 S rRNA gene phylogenetic clades previously designated as A, B, and C (Bhoora et al., 2009; Bhoora et al., 2010a; Rapoport et al., 2014) (Fig. 3).

The published 18 S rRNA sequence from the United States Department of Agriculture (USDA) grouped into clade A (EU642512), while sequences obtained from seven South African isolates (BP3B, BP7, BP9, BP11, BP13, T64, and T73) grouped within clade B together with published sequences from South Africa (EU642513, EU888900, EU888901, EU888904, and Z15104) (Allsopp et al., 1994; Bhoora et al., 2009). The remaining sequence (BP3C), grouped into clade C along with a published South African *B. caballi* sequence (EU642514) (Fig. 3). The seven *B. caballi* 18 S sequences obtained in this study demonstrated a high degree of sequence identity (96.6–100%) with published South African 18 S rRNA gene sequences and 94.4–94.8% identity with the published USDA *B. caballi* 18 S rRNA gene sequence (EU642512) from clade A (Bhoora et al., 2009). Eleven sequences in clade B showed 99.5–100% identity to each other, while the two sequences in clade C showed 99.8% identity to each other. The 18 S rRNA gene sequences in clade C (BP3C) showed between 84.9% and 85.3% identity to sequences occurring in clade B and 94.6% identity to the sequence occurring in clade A.

3.4. The development of the B. caballi typing qPCR assays

3.4.1. The analytical sensitivity and specificity of the typing qPCR assays

The *Bcsbp*4-A, *Bcsbp*4-B, and *Bcsbp*4-C qPCR assays successfully amplified 55 bp, 75 bp, and 75 bp fragments of the *sbp*4 gene, respectively. A linear regression of the *Bcsbp*4-A assay extended from 5.5 $\times 10^{3}$% PE to 5.5 $\times 10^{-4}$% PE with an $R^2>0.99$ and an efficiency of 93%. For the *Bcsbp*4-B assay, the linear regression extended from 5.5 $\times 10^{3}$ to 5.5 $\times 10^{-5}$% PE with an $R^2>0.99$ and an efficiency of 101%. While, for the *Bcsbp*4-C assay, the linear regression ranged from 5.5 $\times 10^{1}$ to 5.5 $\times 10^{-5\%}$ PE with an $R^2>0.99$ and an efficiency of 109% (Fig. 4).

Probit analysis showed that the 95% detection limit of the *Bcsbp*4-A qPCR assay was 1.34 $\times 10^{-2}$% PE, corresponding to a cut-off C_q-value of 35. The 95% detection limit of the *Bcsbp*4-B qPCR assay was lower at 2.2 $\times 10^{-3}$% PE, corresponding to a cut-off C_q-value of 32. While the 95% detection limit of the *Bcsbp*4-C qPCR assay was determined to be the lowest at 2.4 $\times 10^{-4\%}$ PE, corresponding to a cut-off C_q-value of 34 (Fig. 5).

The mean C_q-values and coefficient of variation (CV) observed for

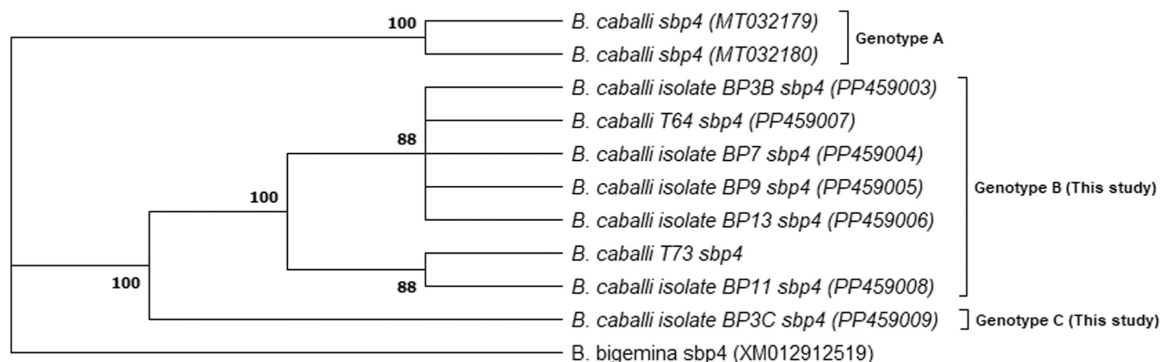

Fig. 2. The Maximum-likelihood phylogenetic tree indicating the evolutionary relationship of *B. caballi* based on the *sbp*4 gene was constructed using MEGA11 (Tamura et al., 2021), applying the Tamura 3-parameter model (Tamura, 1992). Eleven nucleotide sequences, including eight sequences generated from South African isolates in the current study, and a total of 539 positions were considered in the analysis. Clades of *B. caballi* genotypes A, B and C are indicated. Genotype A is represented by published sequences (accession numbers: MT032179 and MT032180). Accession numbers for the remaining sequences obtained in this study (Genotype B and C) are indicated in brackets. The *sbp*4 gene sequence of *B. bigemina* (Accession number: XM012912519) was used as an outgroup. Bootstrap values were based on 1000 replicates.

Fig. 3. The Maximum-likelihood phylogenetic tree indicating tree the evolutionary relationship of *B. caballi* based on the V4 hypervariable region of the 18 S rRNA gene was generated in MEGA11 (Tamura et al., 2021), using the Jukes-Cantor model (Jukes and Cantor, 1969). For tree estimation, 20 nucleotide sequences and 154 positions were considered in the analysis. The 18 S r RNA sequence of *B. divergens* (Z48751) was used as an outgroup. Clades of *B. caballi* genotypes A, B and C are indicated. Accession numbers of published *B. caballi* sequences from South Africa used in the analysis, are indicated in brackets. Bootstrap values were estimated based on 1000 replicates.

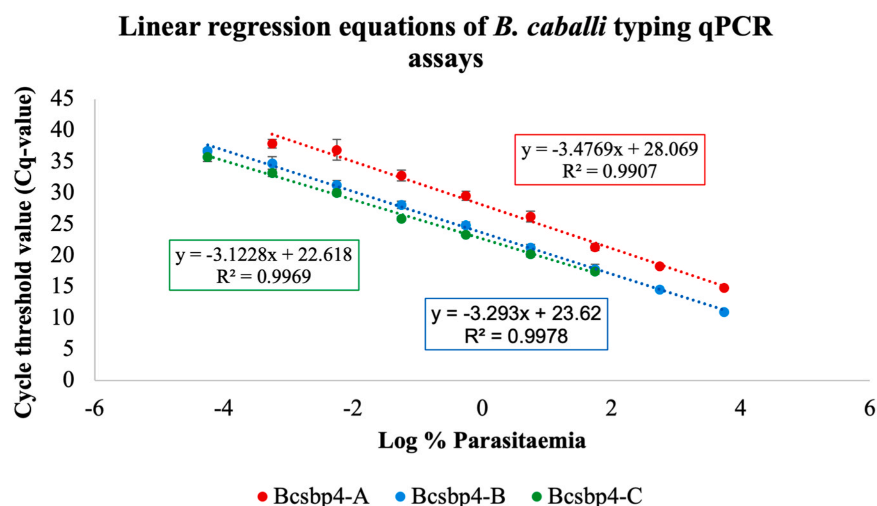

Fig. 4. Linear regression analysis demonstrating the linear range and sensitivity of *B. caballi* typing assays for the detection of genotypes A, B, and C. The cycle threshold (C_q) values were plotted against the log % parasitized erythrocytes (PE) of the 10-fold dilution series of each of the genotype-specific plasmid standards. The amplification range of the molecular typing qPCR assays in detecting genotype A (red), genotype B (blue), and genotype C (green) are indicated.

each *Bcsbp*4 qPCR assay at each dilution is indicated in Table 4. The inter and intra-run standard deviation (SD) for each *Bcsbp*4 assay was low, while the CV between the different replicate runs reached a max of 3.31% for *Bcsbp4*-A qPCR assay; 5.47% for *Bcsbp*4-B qPCR assay and 3.27% for the *Bcsbp*4-C qPCR assay. The decrease in the CV at lower dilutions beyond the detection limit of each assay was noted.

The three *B. caballi* typing qPCR assays were shown to be specific in the detection of their respective genotypes. No cross-reactions between the probes were observed when tested against each of the *B. caballi* genotype-specific plasmid standards or samples containing the related *T. equi* or *T. haneyi* parasites.

3.4.2. Evaluating the efficiency of the B. caballi typing qPCR assays

The efficiency of the *B. caballi* typing qPCR assays was determined by screening field samples (n=18) and *in vitro* cultured isolates (n=5) that were previously confirmed positive for *B. caballi* using the Multiplex-EP qPCR assay. None of the samples tested positive for *B. caballi* genotype A using the *Bcsbp*4-A qPCR assay.

However, genotype B was detected in eight samples using the *Bcsbp*4-B qPCR assay and at C_q-values ranging between 24.4 and 38.3, while the *Bcsbp*4-C qPCR assay detected genotype C in seven samples at C_q-values between 32.2 and 37.8. Samples detected at C_q-values above 39 were considered negative. Neither genotype B nor genotype C could be detected in three (T28, T31, and T32) *B. caballi-positive* field samples. (Table 5). Mixed infections with genotypes B and C were detected in three *in vitro* cultured isolates (BP3, BP9, and BP11), and single infections with genotype B were detected in isolates BP7 and BP13 (Table 5).

A comparison of the level of agreement between the conventional *sbp*4 PCR and the qPCR assays for genotypes B and C was determined using kappa statistics. For genotype B, a kappa value of 0.743 indicated substantial agreement between the two assays, while for genotype C, a fair agreement was observed with a kappa value of 0.326 (Fig. 6).

4. Discussion

Babesia caballi exhibits considerable genetic diversity based on the 18 S rRNA and *rap*-1 genes (Bhoora et al., 2009; Bhoora et al., 2010a; Rapoport et al., 2014). Globally, multiple studies reported that the 18 S rRNA gene sequences of *B. caballi* belong to three phylogenetic clades,

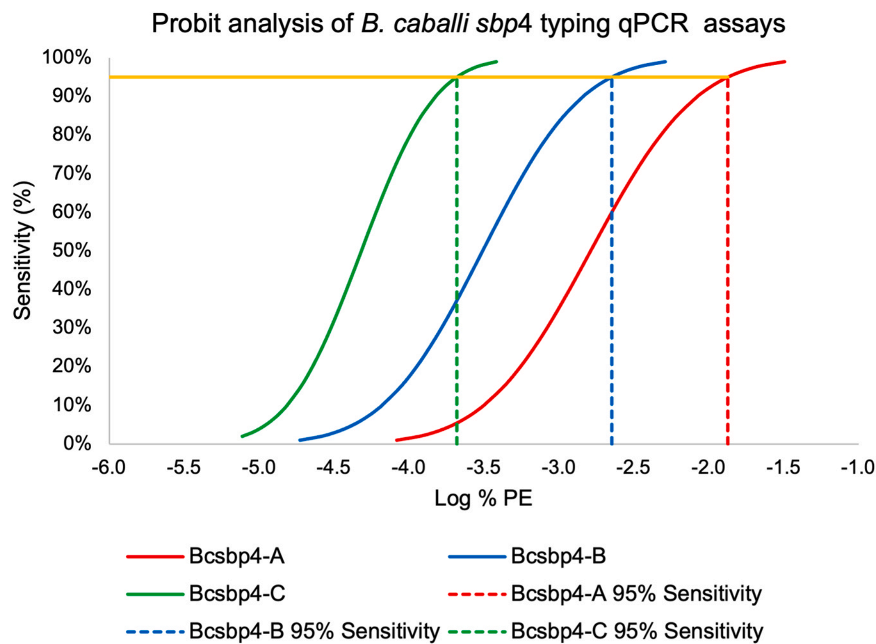

Fig. 5. Probit analysis of the two-fold dilution series of *Bcsbp*4-A (red line), *Bcsbp*4-B (blue line) and *Bcsbp*4-C (green line). The 95% LOD of each genotype-specific assay is indicated through the horizontal (blue) and broken vertical lines.

Table 4
Inter and intra-run variation for eight two-fold dilutions over the non-linear range of each *B. caballi* typing qPCR assay.

*Bcsbp*4-A					
Log %PE	Mean	Inter-run SD	Intra-run SD	Total SD	Total CV%
-1.56	34.63	0.31	0.13	0.44	1.27
-1.86	35.48	0.28	0.29	0.70	1.97
-2.16	35.88	0.38	0.16	0.78	2.18
-2.46	37.18	0.81	0.64	1.06	2.85
-2.77	37.68	0.78	0.00	0.95	2.52
-3.07	37.64	0.62	0.11	0.56	1.48
-3.37	37.74	0.00	0.00	1.25	3.31
-3.67	38.50	0.99	0.00	0.81	2.09
*Bcsbp*4-B					
Log %PE	Mean	Inter-run SD	Intra-run SD	Total SD	Total CV%
-3.56	33.55	0.39	1.06	1.84	5.47
-3.86	33.86	0.40	0.32	1.26	3.71
-4.16	34.45	0.28	0.05	0.69	2.02
-4.46	35.01	0.59	0.00	0.76	2.17
-4.76	35.18	0.70	0.65	1.07	3.04
-5.06	34.41	0.00	0.00	0.06	0.17
-5.36	0.00	0.00	0.00	0.00	0.00
-5.66	33.58	0.00	0.00	0.00	0.00
*Bcsbp*4-C					
Log %PE	Mean	Inter-run SD	Intra-run SD	Total SD	Total CV%
-1.56	34.25	0.12	0.23	0.48	1.41
-1.86	35.13	0.65	0.41	1.15	3.27
-2.16	35.52	0.49	0.25	0.88	2.47
-2.46	35.62	0.41	0.42	0.93	2.62
-2.77	36.18	0.33	0.11	0.33	0.92
-3.07	36.38	1.13	0.00	1.13	3.09
-3.37	36.36	0.00	0.00	0.52	1.42
-3.67	37.35	0.00	0.00	0.00	0.00

Table 5
Comparison of the test results obtained using the *sbp*4-based conventional PCR assays, Multiplex-EP qPCR assay and *B. caballi* typing qPCR assays to screen South African field samples and *in vitro* cultured isolates.

Sample	Conventional		Multiplex-EP	*B. caballi*-typing qPCR results		
	*sbp*4-B (+/-)	*sbp*4-C (+/-)	*B. caballi* (Cq)	*Bcsbp*4-A (Cq)	*Bcsbp*4-B (Cq)	*Bcsbp*4-C (Cq)
T3	-	-	33.7	-	-	36.0
T6	-	-	36.9	-	36.3	-
T12	-	-	35.1	-	-	37.8
T17	-	-	35.9	-	-	37.0
T25	+	-	35.7	-	34.4	-
T27	-	-	34.4	-	-	35.4
T28	-	-	37.6	-	-	-
T31	-	-	36.1	-	-	-
T32	-	-	38.0	-	-	-
T64	+	-	23.5	-	24.4	-
T73	+	-	33.2	-	33.9	-
T75	+	-	31.5	-	31.4	-
T80	-	-	35.2	-	38.3	-
T120	-	-	32.5	-	-	32.5
T122	-	-	32.4	-	-	32.2
T150	-	-	32.5	-	-	34.1
T158	+	-	36.2	-	35.1	-
Z53	-	-	31.5	-	31.1	-
BP3	+	+	16.3	-	17.3	20.7
BP7	+	-	15.5	-	17.3	-
BP9	+	+	19.9	-	20.9	23.1
BP11	+	+	17.0	-	18.7	19.8
BP13	+	-	19.0	-	20.4	-

designated as A, B, and C (Bhoora et al., 2009; Braga et al., 2017; Camino et al., 2020; Manna et al., 2018; Qablan et al., 2013; Sant et al., 2019; Tirosh-Levy et al., 2020; Wang et al., 2019). Two further studies confirmed that *B. caballi rap*-1 gene sequences also clustered into three phylogenetic clades (Bhoora et al., 2010a; Rapoport et al., 2014). However, a recent study in China reported that the *rap*-1 gene sequences clustered into eight clades suggesting that more genetic variation might exist in the *B. caballi* parasites (Wu et al., 2023). The *rap*-1 sequences from South Africa and Israel are grouped in clade 1, consistent with previous findings (Bhoora et al., 2010a; Rapoport et al., 2014).

The high genetic diversity compromises the detection of *B. caballi* using serodiagnostic tests, such as the ELISA and the IFAT, which primarily rely on the binding of antibodies produced by the host's immune system to parasite antigens. This was evidenced by the inability of the

		SBP4-B qPCR		
		Positive	Negative	Total
SBP4-B nested PCR	Positive	10	0	10
	Negative	3	10	13
	Total	13	10	23
Kappa= 0.743				
95% confidence interval: From 0.482 to 1.000				

		SBP4-C qPCR		
		Positive	Negative	Total
SBP4-C nested PCR	Positive	3	0	3
	Negative	7	13	20
	Total	10	13	23
Kappa= 0.326				
95% confidence interval: From 0.019 to 0.634				

Fig. 6. 2×2 contingency tables indicating the level of agreement between the test results obtained using the *sbp*4 conventional PCR (cPCR) and the qPCR in detecting *B. caballi sbp*4-B (A) and *B. caballi sbp*4-C (B) from 23 samples.

commercially available RAP-1 cELISA assay to detect antibodies from all *B. caballi*-infected horses due to the *rap*-1 sequence diversity observed in isolates from South Africa (Bhoora et al., 2010a), Israel (Rapoport et al., 2014), and Egypt (Mahmoud et al., 2016). Improved detection of *B. caballi* infections is therefore necessary.

Several serodiagnostic assays utilizing the SBP4 have been reported for bovine babesiosis (Chung et al., 2017; Mosqueda et al., 2023; Terkawi et al., 2011a). Similarly, a recent study reported the development of an iELISA based on *B. caballi* SBP4 (Mahmoud et al., 2020). The study confirmed the expression of the *Bcsbp*4 gene in erythrocytes of infected horses and its immunogenicity against *B. caballi* (Mahmoud et al., 2020). The *Bc*SBP4 antigen was further shown to be conserved between USDA and Egyptian isolates, that cluster phylogenetically, with *B. caballi* genotype A sequences. However, the ability of the *Bc*SBP4-based iELISA to detect *B. caballi* infections globally has not been investigated (Mahmoud et al., 2020). BLASTP analysis indicated 72.7–76.7% amino acid sequence identities between South African SBP4 protein sequences obtained in this study and published sequences (QMJ54428 and QMJ54429). Consequently, this significant sequence variation suggests that the iELISA may not effectively detect isolates originating from South Africa.

The Multiplex-EP qPCR assay detected *B. caballi* in 18 field samples with C_q-values ranging between 23.5 and 38. Nine field samples had Cq-values > 35, suggesting low infections with *B. caballi.* This may be attributed to the natural clearance of *B. caballi* infections within one to four years, contrasting with the persistent nature of *T. equi* infections throughout the lifespan of an infected horse (de Waal, 1992; Friedhoff et al., 1990; Rothschild, 2013; Wise et al., 2013). Compared to field samples, the C_q-values obtained for *in vitro* cultured isolates were all below 20.

Samples positive for *B. caballi* (n=23; including 18 field samples and 5 *in vitro* cultured isolates) were used to confirm the genetic diversity of the *sbp*4 and 18 S rRNA gene sequences in South Africa. Hence, *Bcsbp*4-B and *Bcsbp*4-C gene-specific primers were designed from the analysis of preliminary whole genome sequence data for *B. caballi* isolates representing genotypes B and C (unpublished data). Using these primers, the *sbp*4 gene was successfully amplified from all five South African *in vitro* cultured isolates and only two field samples. The inability of the conventional PCR assays to successfully amplify the *sbp*4 gene from all field samples could be attributed to the lower parasitaemia, indicated by higher C_q-values for *B. caballi* obtained using the Multiplex-EP qPCR assay (Table 5) (de Waal, 1992).

Amplification using the *sbp*4 genotype-specific primers identified mixed infections of *B. caballi* genotypes B and C in three of the five *in vitro* cultured isolates (BP3, BP9, and BP11), and a sequence representing genotype C was only obtained from one isolate (BP3). Thus, screening more recombinant clones might have resulted in identifying additional sequences representing genotype C.

The phylogenetic analysis of the *sbp*4 and V4 hypervariable region of the 18 S rRNA gene respectively, showed similar clustering. The South African *sbp*4 gene sequences grouped separately from Egyptian (MT032180) and USDA (MT032179) sequences representing genotype A. These results were similar to those reported for the *B. caballi rap*-1 gene sequences, which also grouped South African isolates separately into two clades (B and C) that showed 79–81% identity to the published *rap*-1 gene sequences in clade A (Bhoora et al., 2010a). Thus, the phylogenetic classifications of the South African *B. caballi* isolates based on the *sbp*4, 18 S rRNA and *rap*-1 gene sequences, support the hypothesis that *B. caballi* isolates from South Africa represent two antigenically distinct groups (Bhoora et al., 2010a).

The sequence data obtained in this study enabled the development of molecular typing assays for *B. caballi* targeting the *sbp*4 gene. Each of the newly developed *B. caballi* typing qPCR assays (*Bcsbp*4-A, *Bcsbp*4-B, and *Bcsbp*4-C) showed linearity and specificity in the detection of genotypes A, B, and C, respectively. Furthermore, there was no cross-reaction observed between the different *B. caballi* genotypes. The efficiencies of the three *B. caballi* typing qPCR assays ranged from 93% to 109%, falling within the acceptable range of 90–110% (Kavanagh et al., 2011). The sensitivities of the three *B. caballi* typing qPCR assays were between 10^{-2} to $10^{-4\%}$ PE, which was comparable to that of the *B. caballi* 18 S qPCR assay with a sensitivity of 10^{-4}% PE (Bhoora et al., 2010c, 2018).

The diagnostic ability of the *B. caballi* typing qPCR assays in identifying distinct genotypes was evaluated by screening 18 previously confirmed *B. caballi*-positive field samples. The newly developed assays detected *B. caballi* genotypes B and C in 83% of the tested samples. Genotype B was detected in eight samples, while seven tested positive for genotype C. As previously reported, genotype A was not found in any of the South African field horses (Bhoora et al., 2009, 2010a). Three *B. caballi*-positive field samples could not be genotyped using the *B. caballi* typing qPCR assays or sequenced using conventional PCR assays. This is possibly due to the low parasitaemia levels in these samples as indicated by the higher C_q-values. In contrast, three samples with C_q-values ranging between 32.5 and 34.1, in which the amplification and sequencing of the *sbp*4 gene also proved unsuccessful, were determined to be *B. caballi* genotype C. While the kappa statistics revealed a significant to moderate level of agreement in identifying specific *B. caballi* genotypes with both molecular assays, it is evident that the *B. caballi* qPCR typing assays exhibit higher sensitivity compared to conventional PCR assays for detecting different genotypes in field samples.

A potential avenue for future research involves obtaining additional sequence data from samples, particularly those identified as belonging to the *B. caballi* genotype C group. This would involve optimizing the conventional *sbp*4 PCR assays to ensure successful amplification of the *sbp*4 gene from field samples. Moreover, incorporating the *B. caballi* typing qPCR assays into a multiplex assay for simultaneous identification and quantification of all three genotypes in one reaction would yield benefits like cost savings and enhanced diagnostic efficiency.

Based on the analysis of the amino acid sequences of the *Bc*SBP4 antigen used in the development of the *Bc*SBP4-based iELISA, it is evident that the assay will not detect *B. caballi* infections from South African samples. *In silico* methods can be used to identify conserved immunogenic epitopes within the South African *Bc*SBP4 sequences obtained in this study to design serodiagnostic assays with global significance. However, used in conjunction with the iELISA, the *B. caballi* typing qPCR assays have the potential to enhance diagnostic capabilities

by detecting other genotypes that may otherwise remain undetected.

5. Conclusion

This study reports on the development of *B. caballi* typing qPCR assays that can detect and differentiate between the three known *B. caballi* genotypes (A, B, and C). All three assays had acceptable detection limits and were specific in the detection of the relevant *B. caballi* genotypes from *in vitro* cultured and field samples from South Africa. However, *B. caballi* genotype A has not been identified in South African horses, and it was therefore not possible to evaluate the *Bcsbp*4-A qPCR assay. Quantifying genotype-specific *B. caballi* infections could contribute to understanding the global epidemiology of *B. caballi* genotypes. Additionally, it may help determine whether there is a correlation between specific genotypes, hosts (such as horses, zebras, and donkeys), and the manifestation of clinical disease. Certain *B. caballi* genotypes may exhibit differences in their pathogenicity, interaction with the host, and immune evasion strategies, which can directly impact the clinical outcomes of infected horses. By examining the relationships between genotypes and pathogenicity, we can identify highly virulent strains and create targeted interventions, such as vaccines or treatments, that are specific to these pathogenic genotypes. Moreover, this knowledge can be used to develop risk assessment models, which can help to predict and reduce the impact of particular *B. caballi* genotypes on equine health. Ultimately, understanding the complexities of genotype-associated pathogenicity is critical in improving our capacity to control and prevent the consequences of equine piroplasmosis.

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Animal health and compliance

Ethical approval was obtained from the University of Pretoria Animal and Research Ethics Committees (REC059–22). In addition, permission to conduct the research was obtained from the South African Department of Agriculture, Land Reform and Rural Development under section 20 of the Animals Diseases Act, 1984 (Act No 35 of 1984) with reference number 12/11/1/1/5 (2581BD).

CRediT authorship contribution statement

Raksha Vasantrai Bhoora: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Kgomotso Penelope Sibeko-Matjila:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Investigation, Formal analysis. **Ilse Vorster:** Writing – review & editing, Methodology, Investigation, Data curation. **Nokuzola Faith Nkosi:** Writing – review & editing, Supervision. **Alicia Venter:** Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Raksha Vasantrai Bhoora reports financial support was provided by National Research Foundation. Alicia Venter reports financial support was provided by AgriSETA. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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