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Original article

Quantitative detection of *Theileria haneyi* in South African horses

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ABSTRACT

Theileria haneyi is an apicomplexan parasite closely related to *Theileria equi*, a known causative agent of equine piroplasmosis. The molecular distinction between these parasites relies on a nested polymerase chain reaction (PCR) assay, which has been reported to be unreliable. A recently reported indirect ELISA based on equi merozoite antigen 11 (Th*ema*-11) of *T. haneyi* can detect geographically diverse *T. haneyi* strains. Since the *ema*-11 gene is exclusive to *T. haneyi*, it was chosen as the target for developing a TaqMan minor groove binder (MGB™) quantitative real-time PCR (qPCR). Published *T. haneyi ema*-11 gene sequences were used to design primers to amplify the *ema*-11 gene, and *ema*-11 amplicons from South African samples were cloned and sequenced. An alignment of the South African *ema*-11 gene sequences with published *T. haneyi ema*-11 gene sequences enabled the identification of a conserved region for the design of the qPCR assay. The *T. haneyi ema*-11 (Th*ema*-11) qPCR assay was efficient, specific, and sensitive in detecting *T. haneyi ema*-11. The detection limit was determined to be 1.169×10^{-3} % parasitized erythrocytes. The performance of the Th*ema*-11 qPCR assay was evaluated together with a *T. equi ema*-1-specific qPCR assay. *Theileria haneyi* was detected in 67.6 % of the South African field samples screened, while the occurrence of *T. equi* based on the quantitative amplification of the *ema*-1 gene was higher (91.8 %). Our results suggest that combined, the Th*ema*-11 and *T. equi ema*-1 qPCR assays could detect and differentiate between *T. haneyi* and *T. equi* infections.

1. Introduction

Equine piroplasmosis (EP) is a tick-borne disease of equids caused by the haemoprotozoan parasites, *Theileria equi, Theileria haneyi,* and *Babesia caballi* (Knowles et al., 2018; Mehlhorn and Schein, 1998; Schein, 1988). The disease is endemic in most parts of the world, where tick vectors in the Ixodidae family are present (de Waal, 1992). Almost 90 % of the equine population in the world occurs within endemic regions (Scoles and Ueti, 2015; Tirosh-Levy et al., 2020). In these regions, especially in Africa, the infection rate exceeds 60 %, with most equids suffering from multiple equine piroplasmid infections (Scoles and Ueti, 2015). The international movement of equines is, therefore, strictly regulated to prevent the introduction of EP into areas where the parasites do not naturally occur.

Serological assays for EP, including the complement fixation test (CFT), indirect immunofluorescent antibody test (IFAT), enzyme-linked immunosorbent assay (ELISA), and competitive inhibition ELISA (cELISA) are used to detect positive animals (Laus et al., 2013; Nardini et al., 2022; Wise et al., 2013). However, epidemiological surveys of EP conducted globally identified variant *T. equi* and *B. caballi* parasite genotypes that could not be detected using the WOAH-approved cELISA serological tests for *T. equi* and *B. caballi* (Bhoora et al., 2009, 2020; Chen et al., 2022; Hall et al., 2013; Ketter-Ratzon et al., 2017; Qablan et al., 2013; Salim et al., 2010; Veronesi et al., 2014; Wang et al., 2019; Zhao et al., 2020). Moreover, a borderline positive cELISA and negative *T. equi ema*-1 polymerase chain reaction (PCR) result led to the isolation and characterization of *T. haneyi* from a stray horse captured at the USA-Mexico border near Eagle Pass, Texas (Knowles et al., 2018). *Theileria haneyi* has since been reported to occur in several countries in North America, Africa, and Asia (Bastos et al., 2021; Bhoora et al., 2020; Knowles et al., 2018; Mshelia et al., 2020; Zhou et al., 2023).

Phylogenetic analysis based on the 18S ribosomal RNA and nuclear-encoded protein-coding gene sequences led to the placement of *T. haneyi* within the *Theileria* genus (Knowles et al., 2018). Although the 18S rRNA sequence of this parasite was more closely related to South African *T. equi* 18S genotype C than to the reference *T. equi* Florida strain (Knowles et al., 2018), species classification was also based on differences in the equi merozoite antigen (EMA) multigene family (Knowles

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et al., 2018). *Theileria equi* EMA is encoded by a nine-member multigene family (*ema*1–9) (Kappmeyer et al., 2012). Six of the nine *T. equi* EMA family members (*ema*-2, *ema*-5 to *ema*-9) have orthologs in both species; however, *ema*-1, *ema*-3, and *ema*-4 located on chromosome 1 of *T. equi* do not have orthologs in *T. haneyi*. In contrast, *T. haneyi* has three unique genes coding for EMA, two (*ema*-11 and *ema*-12) found on chromosome 1 and one (*ema*-13) on chromosome 2 (Knowles et al., 2018). The lack of *ema*-1 in *T. haneyi* explains why *T. haneyi* infections cannot be detected by the *T. equi* cELISA assay, which uses a monoclonal antibody specific for EMA-1 (Knowles et al., 2018). The classification of *T. haneyi* as a species separate from *T. equi* was supported by morphological evidence, indicating that *T. haneyi* merozoites appeared much smaller than those of *T. equi* (Knowles et al., 2018).

In contrast to *T. equi*, which causes varying clinical signs, experimental infections with *T. haneyi* showed minimal clinical disease in spleen-intact horses (Knowles et al., 2018; Sears et al., 2019). It was further demonstrated that splenectomized horses infected with *T. haneyi* can clear infections and survive, while *T. equi* infections in splenectomized horses result in fatalities (Sears et al., 2022). Despite the lack of pathogenicity, chemosterilization of horses infected with *T. haneyi* could not be achieved using imidocarb dipropionate (ID) and more notably, ID failed to clear *T. equi* infections in horses co-infected with *T. haneyi* (Sears et al., 2020). Additional *in vivo* studies using tulathrombomycin and diclazuril further indicated that these drugs were also ineffective in the clearance of *T. haneyi* in splenectomized horses (Onzere et al., 2023). This highlights the epidemiological importance of *T. haneyi* as horses are generally co-infected with either *T. equi* and *T. haneyi* or *T. equi, T. haneyi* and *B. caballi* (Bhoora et al., 2020; Coultous et al., 2020; Elsawy et al., 2021; Knowles et al., 2018; Mshelia et al., 2020). Detecting and distinguishing between two or more parasites in the same infected horse is crucial, as co-infections can exacerbate the overall clinical condition and pose challenges for diagnosis and treatment.

The molecular differentiation of *T. equi* and *T. haneyi* has previously been accomplished using two nested PCR assays; the first one targets the *ema*-1 gene of *T. equi* (Baptista et al., 2013), and the second one targets a gene encoding a hypothetical protein of *T. haneyi* that does not have a homolog in *T. equi* (Knowles et al., 2018). These assays confirmed the occurrence of *T. haneyi* in South African horses infected with *T. equi* genotype C; genotype C positive samples were also shown to be infected with *T. equi* or co-infected with both *T. equi* and *T. haneyi*. Additionally, a subset of *T. equi* genotype C positive samples tested negative using the nested PCR, suggesting that the nested PCR is not as sensitive as the qPCR assay (Bhoora et al., 2020; Katjivena, 2022). The results presented in these studies showed that the diagnostic nested PCR assays used to distinguish between *T. haneyi* and *T. equi* genotype C may not be reliable and also strongly suggested that there is an association between *T. haneyi, T. equi ema*-1, and genotype C (Bhoora et al., 2020).

Recently, a *Th*EMA11 indirect ELISA based on recombinant EMA-11 protein was developed to detect *T. haneyi* in the sera of infected horses (Bastos et al., 2021). However, since the specificity of the Th*ema*-11 indirect ELISA was assessed using a panel of serum samples obtained from horses in the USA, Mexico, Puerto Rico, France, Germany, Ireland, and The Netherlands (Bastos et al., 2021; Knowles et al., 2018), possible variations in the EMA-11 protein sequence in other parts of the world may prevent the indirect ELISA from detecting *T. haneyi* globally.

A *T. haneyi* quantitative real-time PCR (qPCR) assay was recently developed to detect *T. haneyi* infections (Zhou et al., 2023). However, false-negative results and a poor correlation between the results of the nested PCR and qPCR assays were reported (Zhou et al., 2023), suggesting that this qPCR assay may not be as specific or as sensitive as the nested PCR in detecting *T. haneyi* infections.

This study aimed to develop a TaqMan minor groove binder (MGB™) qPCR assay based on the *ema*-11 gene to detect *T. haneyi* and better understand this parasite's epidemiology in South Africa.

2. Materials and methods

2.1. Ethical approval, sample collection and DNA extraction

Ethical approval was obtained from the University of Pretoria Animal Ethics Committee (AEC) and Research Ethics Committee (REC) (REC111–21). In addition, permission to perform research in terms of Section 20 of the Animal Diseases Act, 1984 (Act No. 35 of 1984) was obtained from the Department of Agriculture, Land Reform and Rural Development (DALRRD) (Act No 35 of 1984) (12/11/1/1/6 (2156 RJ)).

Blood samples were collected in EDTA tubes from 170 field horses originating from three provinces in South Africa (Table 1). Genomic DNA was extracted from 200 µl of each EDTA blood sample ($n = 170$) using the PureLink® Genomic DNA Mini Kit (Invitrogen™) according to the manufacturer's instructions.

2.2. Nested PCR to detect T. equi and T. haneyi

Nested PCR assays were used to detect *T. equi* and *T. haneyi* positive samples. Detection of *T. equi* was accomplished using the nested PCR assay based on the amplification of the *T. equi ema*-1 gene (Baptista et al., 2013), while *T. haneyi* was amplified using a nested PCR assay targeting a coding region on chromosome 1 of *T. haneyi* that lacks a homolog in *T. equi* (Knowles et al., 2018). The nested PCR assays were performed as previously described (Bhoora et al., 2020).

2.3. Analysis of the T. haneyi ema-11 gene from South African field samples

The published *T. haneyi* Eagles Pass *ema*-11 gene sequence available on GenBank (Accession number: MG652753.1) was used to design PCR primers, ThEMA11_fwd (5′ GGA GAC AGA GAA GCC ATC AAA- 3′) and ThEMA11_rev (5′ CTT TGC CAC CTC CAG AGT ATT 3′), to amplify a 738 bp fragment of the *T. haneyi ema*-11. The primers were evaluated *in silico* for their specificity using BLASTn (Altschul et al., 1990). The primers were used to amplify the 738 bp fragment of the *T. haneyi ema*-11 gene from samples identified as *T. haneyi* positive using the nested PCR assay. The PCR reactions were prepared in a total volume of 25 µl comprising 1X DreamTaq Green PCR Master mix, 0.2 µM of each ThEMA11_fwd and ThEMA11_rev primer (Inqaba Biotechnologies, South Africa), and 5 µl of genomic DNA. The cycling conditions included an initial denaturation of 3 min at 95 °C, followed by 30 cycles of 30 s at 95 °C, 30 s at 55 °C and 1 min at 72 °C, and a final extension of 7 min at 72 °C. All amplicons were visualised by electrophoresis on a 2 % agarose gel.

Theileria haneyi ema-11 amplicons were purified using the PureLink® Quick Gel and PCR Purification kit (Invitrogen™) and cloned into the pJET1.2/blunt cloning vector using the sticky-end protocol of the CloneJET™ PCR Cloning kit (ThermoFisher Scientific™, South Africa) according to the manufacturer's instructions. Three recombinant clones per sample were selected, plasmid DNA was isolated using the GeneJet Plasmid Miniprep kit (ThermoFisher Scientific™, South Africa) and plasmid inserts were sequenced at Inqaba Biotechnologies (South Africa) using the pJET1.2 forward and reverse sequencing primers. Sequences were assembled and edited using Gap4 of the Staden software

Table 1
Origin and number of equine blood samples obtained for analysis.

Geographical Area	Province	No. of samples
Vryheid	KwaZulu-Natal	30
Tafuleni	KwaZulu-Natal	48
Ntembeni	KwaZulu-Natal	32
SummerVeld	KwaZulu-Natal	6
Ganyesa	North-West	36
Pretoria East	Gauteng	11
Equine Clinic, University of Pretoria	Gauteng	7
Total		170

suite (Staden et al., 2000). The sequences were trimmed and aligned using the BioEdit Sequence Alignment Editor (Hall, 1999). The *T. haneyi ema*-11 gene sequences were deposited in GenBank under accession numbers PP706122 to PP706133.

BLASTn was used to search the public sequence databases for homologous sequences (Altschul et al., 1990), and the *T. haneyi ema*-11 gene sequences were aligned using MEGA X (Kumar et al., 2018). MLModelTest (MEGA X) was used to select a Kimura 2-parameter substitution model with a proportion of invariable sites (K2 + *I*) and a gamma-shaped distribution of rates across sites. A maximum likelihood phylogenetic tree with 1000 bootstrap replicates was constructed using a *T. equi ema*-1 sequence (accession number: MF447154) as an outgroup (Kimura, 1980).

2.4. Design of a Thema-11-specific qPCR assay

Real-time primer and probe sequences (Table 2) were designed using Primer Express 3.0.1 (Applied Biosystems™) to amplify and detect a 69 bp fragment within a conserved region identified in the *ema*-11 gene sequence alignment. The ThEMA11_qFWD primer was designed to contain two degenerate bases and the TaqMan (MGB™) probe was labelled with a VIC fluorescent reporter dye at the 5′-end. Primers and probes were synthesized by ThermoFisher Scientific™, South Africa. An *in silico* BLAST analysis of the primer and probe sequences was performed to determine potential cross-reactions with known sequences in the public sequence databases.

The Th*ema*-11 qPCR assay was conducted using the QuantStudio™ 5 Real-time PCR instrument (ThermoFisher Scientific™, South Africa). Reactions (20 µl) contained 10 µl KAPA probe Fast Universal Master Mix (KAPA Biosystems), 0.9 µM of each of the ThEMA11 forward and reverse primers, 0.25 µM of the TaqMan MGB™ probe, and 2.5 µl of target DNA. The cycling conditions included an initial denaturation of 20 s at 95 °C, followed by 40 cycles of 1 s at 95 °C and a final extension of 30 s at 60 °C.

Table 2
Oligonucleotide primer and probe sequences used in the real-time PCR assays in this study.

Primer/Probe	Sequence 5’ -3’	Reference
Th*ema*-11-specific qPCR assay		This study
ThEMA11_qFWD	GCC ACA YGT YTT GGT TAC TGT TG	
ThEMA11_qREV	ACC CAC TTG TCA CCA TCC TTC T	
ThEMA11_qPROBE	VIC-CCT GCT CTT CGT CTT G-MGB	
Multiplex-EP qPCR		Bhoora et al. (2018)
Be18SF	GCG GTG TTT CGG TGA TTC ATA	
Be18SR	TGA TAG GTC AGA AAC TTG AAT GAT ACA TC	
Be18SP	FAM-AAA TTA GCG AAT CGC ATG GCT T-QSY	
Bc_18SF402	GTA ATG GAA TGA TGG CGA CTT AA	
BC_18SR496	CGC TAT TGG AGC TGG AAT TAC C	
BC_18SP	VIC-CCT CGC CAG AGT AA-MGB	
T. equi ema-1-specific qPCR assay		Bhoora et al (2010)
RT_EMAF	CCG GCA AGA AGC ACA YCT T	
RT_EMAR	TGC CAT CGA CGA YCT TGA G	
RT_EMAprobe	6-FAM-TCC AGG CAA GCG C-MGB	
Multiplex equine *Theileria* genotyping qPCR assays		Bhoora et al. (2020)
TeGeno_fwd	ACC TTA ACC TGC TAA ATA GG	
TeGeno_rev	GCT TCT TAG AGG GAC TTT GC	
TeGtPrA	6-FAM-TGT GAG ACT TGG TTT CAT TTC C-MGB	
TeGtPrB	VIC-ATG TGA GAT TCG GTC TCA CCA-MGB	
TeGtPrC	NED-TGT TGG AGT TAT GTT CTA CAC T-MGB	
TeGtPrD	6-FAM-TGT TGG ATT CTA GTT CTA CAC T-MGB	
TeGtPrE	VIC-ATG CGA GAT TTG GTC TCG TTA-MGB	

2.5. Amplification efficiency, sensitivity and specificity of the Thema-11 qPCR assay

The analytical sensitivity of the Th*ema*-11 qPCR assay was determined using a GeneArt plasmid construct (ThermoFisher Scientific™, South Africa) containing the targeted *T. haneyi ema*-11 gene fragment. The plasmid DNA concentration was quantified using a Xpose™ spectrophotometer (Trinean, Belgium). Based on the calculated copy number, the concentration of the plasmid construct was adjusted to 10^9 copies/µl and the genomic equivalent per µl of plasmid DNA was converted into % parasitaemia (% parasitized erythrocytes; PE) by assuming an average of 9×10^6 red blood cells per µl of equine blood. A ten-fold serial dilution (5.5×10^3 to 5.5×10^{-6} % PE) was prepared, and qPCR amplification of the standard dilution series was repeated in triplicate and on five separate occasions. The data generated from each of the 15 runs was used to calculate linear regression equations of the quantification cycle (Cq) (Bustin et al., 2009) against the log % PE, from which the amplification efficiency of the assay was determined. Probit analysis was conducted over the non-linear range of the assay to determine the limit of detection (LOD) of the assay. Five replicates of a two-fold serial dilution starting at 5.5×10^{-2} % PE were prepared. Each dilution series consisted of 11 separate dilutions and each replicate was tested on three separate occasions. The sensitivity (%) of the assay was plotted against the log % PE, and the 95 % detection limit was calculated using the Statistical Package for the Social Sciences Analysis (SPSS Statistics v25, IBM Analytics, USA).

The specificity of the Th*ema*-11 qPCR assay was tested using DNA isolated from *T. equi* and *B. caballi* isolates cultured *in vitro* at the Department of Veterinary Tropical Diseases, University of Pretoria (DVTD, UP).

2.6. Detection of equine Theileria species and B. caballi in field samples

2.6.1. Detection of T. haneyi using the Thema-11 qPCR assay

The effectiveness of the Th*ema*-11 qPCR assay in detecting *T. haneyi ema*-11 was evaluated by screening all field samples ($n = 170$).

2.6.2. Detection of equine piroplasmid species using the Multiplex-EP qPCR assay

The field samples were screened for equine piroplasms using a published Multiplex-EP real-time PCR assay (Bhoora et al., 2018). It should be noted that the “*T. equi*” 18S rRNA gene-specific probe included in the Multiplex-EP real-time PCR assay will detect both *T. equi* and *T. haneyi*. The Multiplex-EP real-time PCR assay was adapted for use on the QuantStudio™ 5 Real-time PCR instrument (ThermoFisher Scientific™, South Africa), with minor modifications to the probes (Table 2). Briefly, the *T. equi* probe was labelled with a fluorescent FAM (6-carboxyfluorescein) reporter dye at the 5′-end and a nonfluorescent quencher (QSY) at the 3′-end and the *B. caballi* probe was labelled with a fluorescent VIC reporter dye at the 5′-end and a nonfluorescent minor groove binder (MGB) at the 3′-end. Reactions were performed in MicroAMP optical 96-well reaction plates as previously described (Bhoora et al., 2018). GeneArt plasmid constructs (ThermoFisher Scientific™, South Africa) containing the qPCR target regions of either *T. equi* (127 bp) or *B. caballi* (159 bp) were synthesized and used as positive controls, while nuclease-free water was used as a negative control.

2.6.3. Detection of T. equi using the T. equi ema-1 qPCR assay

The *T. equi ema*-1 qPCR assay was performed as previously described (Bhoora et al., 2010). Briefly, primers RT-EMA1F and RT-EMA1R, in combination with a FAM-labelled TaqMan MGB™ probe, RT-EMA (Table 2), were used to detect a 59 bp fragment of the *ema*-1 gene of *T. equi*. The reactions were prepared and the amplification was performed as previously described (Bhoora et al., 2010), except that a QuantStudio™ 5 Real-time PCR instrument was used.

2.7. Comparison of nested PCR assays and qPCR assays for detection of T. equi and T. haneyi

The Kappa coefficient was used to determine the level of agreement between the nested PCR assays and the qPCR assays for detecting *T. equi* and *T. haneyi*, respectively.

2.7.1. Molecular genotyping of Theileria positive samples

Samples that were identified as *Theileria* positive using the real-time PCR assays ($n = 158$) were genotyped using the molecular genotyping assays developed for *T. equi* (Bhoora et al., 2020), with a minor modification. Genotypes A and B were detected using the Multiplex EP-AB assay, whilst genotypes C, D and E were detected using the Multiplex EP-CDE assay (Table 2). Quantitative real-time assays were conducted using the QuantStudio™ 5 Real-time PCR instrument (ThermoFisher Scientific™, South Africa). Reactions were performed as previously described (Bhoora et al., 2020). GeneArt plasmid constructs (ThermoFisher Scientific™, South Africa) representing each *T. equi* genotype were synthesized and used as positive controls. Data was analysed using the QuantStudio™ Design and Analysis software version 2.6.0 (ThermoFisher Scientific™, South Africa).

3. Results

3.1. Nested PCRs to confirm T. haneyi and T. equi infections

To identify *T. haneyi* infections, 170 field samples were screened using nested PCR assays specific for *T. haneyi* (Knowles et al., 2018) and *T. equi* (Baptista et al., 2013). A total of 85 samples tested positive for *T. haneyi*, and 131 samples tested positive for *T. equi*. Fourteen samples were positive for *T. haneyi* only, 60 samples tested positive for *T. equi* only, 71 had mixed infections with both parasites, and 25 were negative (Fig. 1).

3.2. Characterization of the T. haneyi ema-11 gene

Primers ThEMA11_fwd and ThEMA11_rev were designed to amplify a 738 bp fragment of the *T. haneyi ema*-11 gene, and BLASTn analysis revealed that the primers were specific for *T. haneyi ema*-11 sequences.

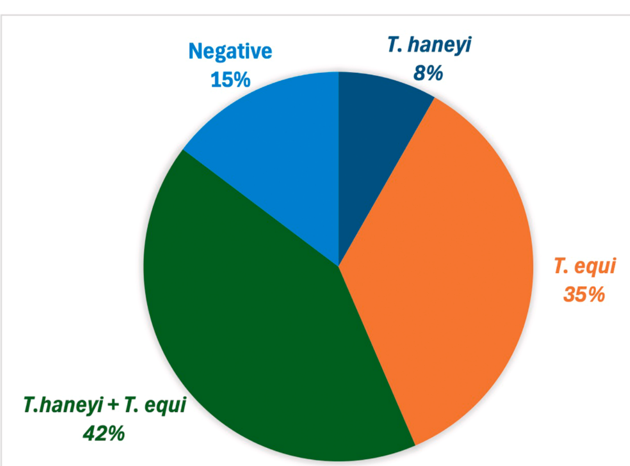

Fig. 1. Pie chart illustrating the distribution of *T. equi* and *T. haneyi* DNA detected in field blood samples using nested PCR assays. The chart categorises the samples as follows: positive for *T. haneyi* (8 %), positive for *T. equi* (35 %), positive for both *T. equi* and *T. haneyi* (mixed infections) (42 %), and negative results (15 %).

An amplicon of the expected size was generated from 60 of the 85 samples identified as nested PCR positive for *T. haneyi*.

Ten *T. haneyi ema*-11 positive samples (T4, T6, T11, T15, T94, T98, T127, T134, T135, T138) were randomly selected for cloning and sequencing of the 738 bp fragment of the *T. haneyi ema*-11 gene. An insert of the correct size was obtained from 15 recombinant clones from nine of the *T. haneyi* positive samples. Sequences generated from the clones were subjected to BLASTn analysis, which indicated 89–99 % sequence identity to the published *T. haneyi ema*-11 gene sequence (accession number: MG652753). A multiple sequence alignment of the 15 South African *T. haneyi ema*-11 gene sequences with the published *T. haneyi ema*-11 gene sequence revealed variable regions between the sequences, and conserved regions suitable for the design of primers and a probe for the Th*ema*-11 qPCR assay were identified (Fig. 2).

A maximum likelihood phylogenetic tree showing the relationships between *T. haneyi ema*-11 sequences revealed that the *T. haneyi ema*-11 sequences fall within two main phylogenetic groups (Fig. 3). The published *T. haneyi ema*-11 sequence was most closely related to group 2 sequences, while only South African *T. haneyi ema*-11 sequences were found in group 1. Sequences from South African field samples T4 and T11 were represented in both groups.

3.3. Analytical efficiency, sensitivity and specificity of the Thema-11 qPCR assay

The *in silico* blast analysis of the qPCR primer and probe sequences revealed 2 to13 nucleotide differences in the forward and reverse primer sequences compared to published *T. equi ema*1–9 sequences. However, the Th*ema*11_qPROBE sequence showed 100 % identity not only with the published *T. haneyi* Eagles Pass *ema*-11 (MG652753) but also with the *T. equi* strain WA *ema*-3 (XM_004830186) gene sequences.

The Th*ema*-11 qPCR assay successfully detected a 69 bp region of the *T. haneyi ema*-11 gene. The linear regression of the assay extended from 5.5×10^3 to 5.5×10^{-4} % PE with an $R^2 > 0{,}99$ and an efficiency of 106 % (Fig. 4A). No amplification was observed from DNA isolated from *T. equi* or *B. caballi in vitro* culture isolates (results not shown), indicating that the assay is specific (Fig. 4B).

A two-fold-serial dilution was prepared to cover the non-linear range of the developed Th*ema*-11 qPCR assay. *Theileria haneyi* could be detected in all 15 replicates of the two-fold serial dilutions ranging from 5.5×10^{-2} % PE to 1.7×10^{-4} % PE (Fig. 4C). Probit analysis revealed that the detection limit of the assay, defined as the concentration at which 95 % of known positive samples can be detected, was 1.5×10^{-3} % PE equating to a cut-off Cq value of 34. The sensitivity of the assay in detecting *T. haneyi* decreased at higher dilutions from 86 % (8.6×10^{-4} % PE) to 13 % (5.4×10^{-5} % PE). The mean Cq values and the coefficient of variation observed for *T. haneyi* at each dilution are shown in Table 3. The *T. haneyi ema*-11 gene intra- and inter-run standard deviations (SD) were low, while the coefficient of variation (CV) between the different replicates reached a maximum of 3.34 %, indicating low variation between the replicate runs.

3.4. Detection of equine Theileria species and B. caballi in field samples

We screened 170 field samples using the newly developed Th*ema*-11 qPCR assay. Of these, 115 samples (67.6 %) tested positive for *T. haneyi ema*-11 with Cq values less than 40. For 102 samples, the Cq values ranged from 26.3 (3.7×10^{-1} % PE) to 33.9 (1.5×10^{-3} % PE), while 13 samples had Cq values above the 95 % Cq cut-off value of 34, and 55 samples tested negative for *T. haneyi* (Fig. 5).

The field samples were further screened using the Multiplex-EP real-time PCR assay (Bhoora et al., 2018) and the *T. equi ema*-1 qPCR assay (Bhoora et al., 2010). Using the Multiplex-EP real-time PCR assay, *Theileria* species infections were detected in 156 of the field samples, and *B. caballi* was detected in 19 samples. Single *Theileria* infections were detected in 140 samples and single *B. caballi* infections in three samples.

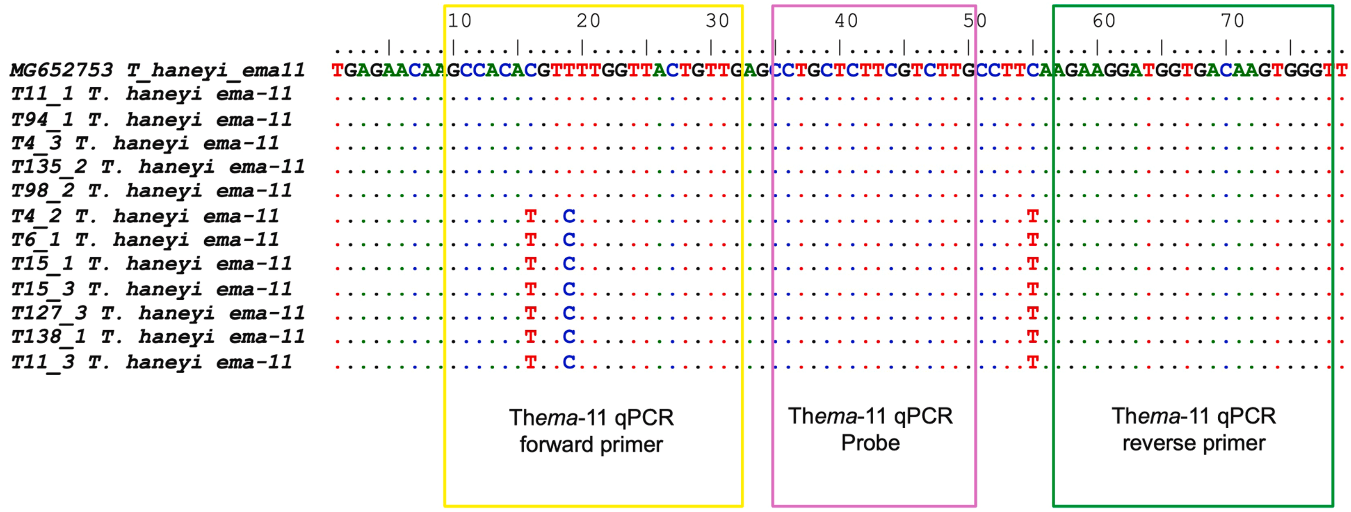

Fig. 2. Nucleotide sequence alignment of the *T. haneyi* ema-11 gene sequences obtained in this study with the published *T. haneyi* ema-11 gene sequence (accession number: MG652753). The positions of the *T. haneyi* ema-11 real-time forward primer (yellow box), the species-specific probe (pink box) and the reverse primer (green box) are indicated. Identical nucleotide positions in the alignment are represented by a period (.).

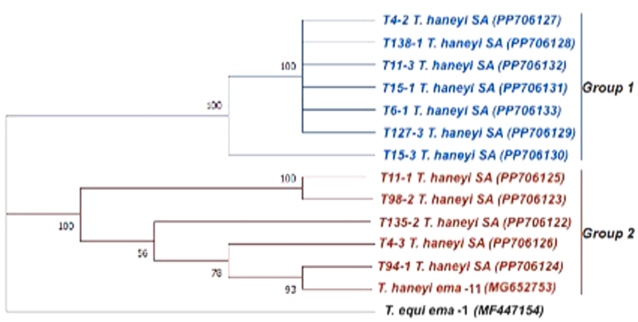

Fig. 3. Maximum likelihood phylogenetic tree showing the relationships between *T. haneyi* ema-11 gene sequences. Accession numbers for the *T. haneyi* ema-11 gene sequences are indicated in brackets. The evolutionary history was inferred using the Kimura 2-parameter model (K2+*I*) (Kimura, 1980). Bootstrap values were estimated based on 1000 replicates. Theileria equi ema-1 (accession number: MF447154) was used as an outgroup. There was a total of 731 positions in the final dataset. The evolutionary analyses were conducted in MEGA X (Kumar et al., 2018).

Sixteen samples had mixed *Theileria* and *B. caballi* infections, and eleven samples were negative (Data not shown).

Using the *T. equi ema*-1 qPCR assay, 156 samples (91.8 %) tested positive.

A total of 158 samples tested positive for equine *Theileria* parasites using either the equine *Theileria* 18S rRNA gene-specific qPCR included in the Multiplex-EP real-time PCR assay (equine *Theileria* 18S qPCR), the *T. equi ema*-1 qPCR assay or the Th*ema*-11 qPCR assay (Fig. 6A). Although the equine *Theileria* 18S qPCR and the *T. equi ema*-1 qPCR assay each detected 156 positive samples, 154 samples tested positive with both assays. Two samples tested negative using the equine *Theileria* 18S qPCR but positive with the *T. equi ema*-1 qPCR assay, which might suggest that the *T. equi ema*-1 qPCR is a more sensitive test. It is possible, however, that these were false positive results, especially as these two samples could not be genotyped. A further two samples tested positive using the equine *Theileria* 18S qPCR but negative with the *T. equi ema*-1 qPCR assay; one of these was positive using the Th*ema*-11 qPCR assay, suggesting that this sample contained a single *T. haneyi* infection. All 115 samples that tested positive using the Th*ema*-11 qPCR assay also tested positive for equine *Theileria* using the 18S qPCR. A total of 114 samples (67.1 %) tested positive with all three tests, suggesting that these samples contained mixed *T. haneyi* and *T. equi* infections. A further 42 samples tested positive using the *T. equi ema*-1 qPCR assay, 40 of which also tested positive with the equine *Theileria* 18S qPCR, suggesting that these samples were infected with *T. equi* only. Our results suggest that the Th*ema*-11 qPCR assay and the *T. equi ema*-1 qPCR assay are able to detect and differentiate between *T. haneyi* and *T. equi* infections.

Mixed *T. haneyi* and *T. equi* infections were common and occurred in samples from all three provinces examined (Fig. 6B). While single infections with *T. equi* were identified in all three provinces, only one sample from Gauteng was identified with a single *T. haneyi* infection. It should be noted, however, that this sample was also infected with *B. caballi* (data not shown).

3.5. Comparison of nested PCR assays and qPCR assays for detection of *T. equi* and *T. haneyi*

The results of the nested PCR assays for detecting *T. haneyi* (Knowles et al., 2018) and *T. equi* (Baptista et al., 2013) were compared to those obtained using the Th*ema*-11 qPCR assay and the *T. equi ema*-1 qPCR assay (Bhoora et al., 2010) (Table 4). A comparison of the agreement between the *T. haneyi* nested PCR assay and the Th*ema*-11 qPCR, as determined using kappa statistics, indicated a moderate agreement between the two tests with a kappa value of 0.506 (Table 4A). The number of observed agreements between the two tests represented 75 % of the observations. For the detection of *T. equi*, a kappa value of 0.420 indicated a moderate agreement between the *T. equi ema*-1 nested PCR assay and the *T. equi ema*-1 qPCR assay, with the number of observed agreements representing 84 % of the total observations (Table 4B). Considerably more samples tested positive with the qPCR assays than the nested PCR assays, although six samples that tested positive with the *T. haneyi* nested PCR tested negative with the Th*ema*-11 qPCR assay, and one sample that tested positive with the *T. equi* nested PCR tested negative with the *T. equi ema*-1 qPCR assay.

3.6. *Theileria* genotyping

The *Theileria* genotypes in 155 of the 170 field samples could be determined using the Multiplex-EP AB and Multiplex-EP CDE assays (Bhoora et al., 2020); one sample that tested *Theileria* positive using the Multiplex-EP real-time PCR assay could not be genotyped. Genotypes A (present in 78.8 % of samples) and C (present in 77.6 % of samples) were most frequently observed, while genotype D occurred in 17.1 % of

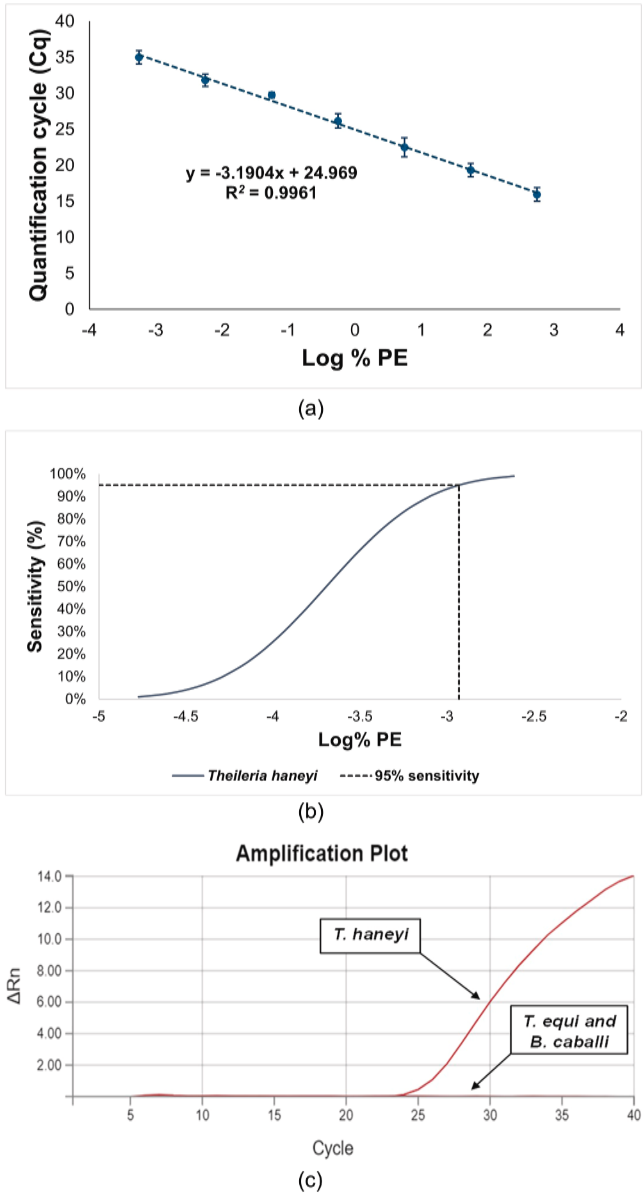

Fig. 4. **(A)** Standard curve for the quantification of the *T. haneyi ema*-11 gene, showing quantification cycle (Cq) plotted against log % PE (parasitized erythrocytes) of a tenfold dilution series of *T. haneyi* DNA equivalent to 5.5×10^{3} to 5.5×10^{-4} % PE. **(B)** Probit analysis of a two-fold dilution series of *T. haneyi* DNA equivalent to 5.5×10^{-2} % PE to 1.7×10^{-4} % PE covering the non-linear range of the assay. The detection limit (95 % sensitivity) is indicated by a dotted line. **(C)** Detection of *T. haneyi* positive control DNA using the Th*ema*-11 qPCR assay, indicated by an increase in the fluorescence signal. No increase in fluorescence signal was observed from *T. equi* and *B. caballi* positive control DNA.

samples. Although single infections of genotypes A, C and D were identified, mixed infections with A and C (55.3 % of samples), A and D (2.9 %), C and D (2.9 %), and A, C and D (10.6 %) were common (Fig. 7A).

The Th*ema*-11 qPCR only detected *T. haneyi* in samples that were positive for equine *Theileria* genotype C, either as a single genotype infection or in combination with other genotypes (AC, CD and ACD) (Fig. 7B). Similarly, the *T. haneyi*-specific nested PCR also only detected *T. haneyi* in samples that were positive for genotype C (data not shown). Samples that were positive for genotypes A ($n = 17$), D ($n = 1$) and AD ($n = 5$) only tested positive with the *T. equi ema*-1 qPCR assay (Fig. 7B); *T. haneyi* was not detected in these samples by either nested PCR or qPCR.

Table 3
Inter- and intra-run variation for 11 twofold dilutions of the T. haneyi ema-11 plasmid prepared over the non-linear range of the assay.

Log 10 dilution	Mean Cq	Inter-run SD	Intra-run SD	Total SD	Total CV (%)
-1.5	30.66	0.34	0.34	0.34	1.11
-1.8	31.98	0.44	0.55	0.44	1.39
-2.1	32.83	0.64	0.66	0.64	1.95
-2.4	33.46	1.01	0.75	1.01	3.02
-2.7	34.55	1.05	0.61	1.05	3.07
-3.0	35.64	1.09	0.96	1.09	3.08
-3.3	35.39	1.11	0.79	1.11	3.34
-3.6	35.72	0.96	0.80	0.96	2.70
-3.9	35.50	0.83	1.02	0.83	2.36
-4.2	36.90	0.66	0.00	0.66	1.80
-4.5	0.00	0.00	0.00	0.00	0.00

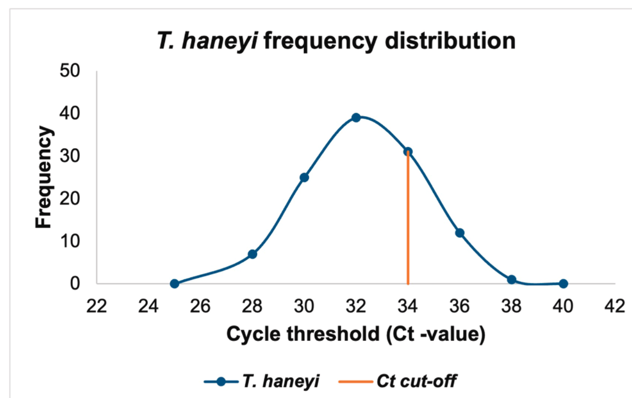

Fig. 5. Frequency distribution of the cycle threshold (Ct) values from all field samples tested with the Th*ema*-11 qPCR assay. The blue line corresponds to the frequency distribution of Ct values observed for *T. haneyi* positive field samples. The orange line represents the detection limit (95 % sensitivity) of the assay in detecting *T. haneyi* DNA.

4. Discussion

Effective methods for controlling and preventing equine piroplasmosis depend on accurate detection of *T. equi, T. haneyi,* and *B. caballi* parasites. The application of accurate and sensitive detection assays ensures the identification of infected horses, enabling the implementation of appropriate management strategies. This includes administering effective treatment to prevent the spread of these parasites to uninfected animals. Furthermore, identification of asymptomatic carrier animals is essential to prevent the international movement of infected horses from endemic to non-endemic regions.

Our study aimed to develop a *T. haneyi ema*-11-specific qPCR assay to better understand the epidemiology of the *T. haneyi* parasite in South Africa. The *ema*-11 gene was chosen as the target of the qPCR assay since the EMA-11 protein is exclusively found in *T. haneyi* (Knowles et al., 2018) and has previously been demonstrated to be highly conserved among several geographically distinct samples (Bastos et al., 2021). In order to develop the qPCR assay, *ema*-11 gene sequences were generated from nine South African field samples. The South African *ema*-11 gene sequences showed 89–99 % sequence identity to the published *T. haneyi ema*-11 gene sequence (MG652753) and confirmed the presence of distinct *T. haneyi ema*-11 sequences in South Africa. Phylogenetic analysis revealed that the *ema*-11 gene sequences were distributed between two groups. Sequences in group 2, exhibiting 96–99 % identity to the published *T. haneyi ema*-11 gene sequence (MG652753), demonstrated higher conservation and lower diversity compared to group 1 sequences,

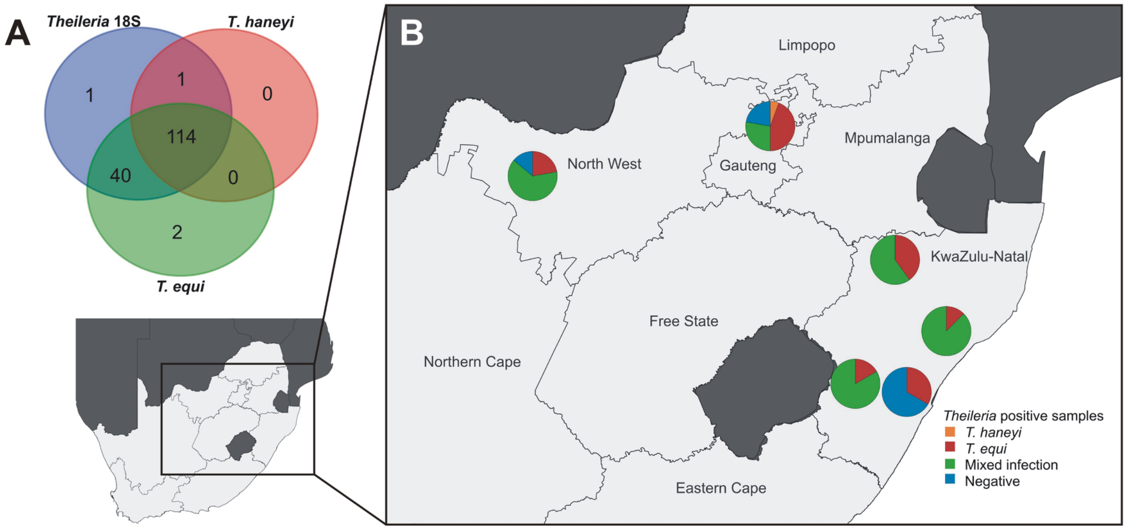

Fig. 6. **(A)** Number of field samples that tested positive for equine *Theileria* parasites using the equine *Theileria* 18S rRNA gene-specific qPCR included in the Multiplex-EP real-time PCR assay, the *T. equi ema*-1 qPCR assay and the Th*ema*-11-specific qPCR assay. Of the 170 field samples examined, a total of 158 samples tested positive using either one, two or all three of the tests. The Venn diagram was created using the online tool available at https://bioinformatics.psb.ugent.be/cgi-bin/liste/Venn/calculate_venn.htpl. **(B)** Geographical distribution of *T. equi, T. haneyi* and mixed equine *Theileria* species infections identified in 170 field samples from three provinces in South Africa. The map was created using the Free and Open Source QGIS.

Table 4
2X2 contingency tables indicating the agreement level between the nested and qPCR assays in detecting either (A) T. haneyi or (B) T. equi from the 170 field samples.

A: Detection of *T. haneyi*				
		T. haneyi ema-11 qPCR		
		Positive	Negative	Total
T. haneyi nested PCR	Positive	79	6	85
	Negative	36	49	85
	Total	115	55	170
Kappa = 0.506 *				
B: Detection of *T. equi*				
		T. equi ema-1 qPCR		
		Positive	Negative	Total
T. equi nested PCR	Positive	130	1	131
	Negative	26	13	39
	Total	156	14	170
Kappa = 0.420 *				

* Indicates moderate agreement

which showed 88–89 % identity to the published *T. haneyi ema*-11 gene sequence (MG652753). It should be noted that *ema*-11 gene sequences were obtained from only nine samples, and it will be advisable to screen more samples to ascertain whether there is more variation in the South African *T. haneyi ema*-11 gene sequences than what has been determined. An analysis of the translated EMA-11 sequences obtained in this study to the published EMA-11 sequence (Wise et al., 2019) indicated amino acid identities ranging from 75.1 % to 82.6 %. These amino acid differences could impact the efficiency of the published Th*ema*-11 indirect ELISA assay in detecting *T. haneyi* infections in South African equids.

A TaqMan minor groove binder (MGB™) quantitative real-time PCR (qPCR) assay was developed to amplify and detect the *ema*-11 gene from *T. haneyi*. Degenerate nucleotide bases were included in the forward primer sequence to enable the detection of all known *ema*-11 gene sequences identified in this study. The Th*ema*-11 qPCR assay developed in this study showed excellent linearity with high analytical sensitivity and specificity. No cross-reactivity was observed when DNA from *T. equi* and *B. caballi* were tested. Even though the Th*ema*-11_qPROBE sequence demonstrated 100 % identity with *T. equi ema*-3, these results demonstrate that the nucleotide differences in the forward and reverse primers were sufficient to prevent amplification and detection of *ema*-3. The reported detection limit (95 % sensitivity) of the assay, 1.2×10^{-3} % PE at a Cq cut-off value of 34, is lower than the detection limits previously reported for the multiplex-EP qPCR assay (1.4×10^{-4} % PE for *T. equi* and 2.8×10^{-4} % PE for *B. caballi*), and the *T. equi ema*-1 qPCR assay (1.4×10^{-4} % PE) (Bhoora et al., 2010, 2018). Several factors can affect the efficiency of an assay, including PCR inhibitors, contaminants, primer sequences, PCR reagents, purity, and sample (Nolan and Bustin, 2013). Amplification efficiencies higher than 110 % can impact the calculated detection limits and Cq cut-off values of the assay, resulting in false-negative results.

Without a set of gold standard positive samples, it is difficult to determine whether the tests can distinguish between *T. haneyi* and *T. equi* parasites. However, specificity testing indicated that the Th*ema*-11 qPCR assay did not detect DNA extracted from *in vitro* cultured isolates of *T. equi* or *B. caballi*. Furthermore, one field sample that tested positive using the equine *Theileria* 18S qPCR also tested positive only with the Th*ema*-11 qPCR assay, and 40 samples that tested positive with the equine *Theileria* 18S qPCR were also positive only for *T. equi ema*-1, suggesting that the assays could distinguish between the two parasites. Evaluation of the Th*ema*-11 qPCR assay, together with the *T. equi ema*-1 qPCR assay on 170 field samples, confirmed the presence of *T. haneyi ema*-11 and *T. equi ema*-1 positive samples in South Africa. The Th*ema*-11 qPCR assay detected *T. haneyi* in 115 field samples, 30 more than detected using the *T. haneyi*-specific nested PCR assay. The *T. equi ema*-1 qPCR assay detected *T. equi* in 156 field samples, indicating a higher sensitivity than the *T. equi*-specific nested PCR assay, which only detected *T. equi* in 131 samples. The discrepancies observed between the nested qPCR assays and the qPCR assays could be attributed to the inherent heterogeneity in the surface-exposed antigenic *ema* genes, which are under intense selection pressure. This sequence variability is expected and warrants further investigation into the complete set of *ema* genes in both *T. equi* and *T. haneyi* in South Africa.

Multiplex-EP real-time assay results indicated that although some field horses were infected with equine *Theileria* species and *B. caballi* simultaneously (9.4 %), most horses were primarily infected with *Theileria* species (82.3 %). Our findings agree with previous studies in South Africa that reported a higher occurrence of *T. equi* than *B. caballi* in field horses (Bhoora et al., 2018; Katjivena, 2022; Motloang et al., 2008). The low percentage of *B. caballi* infections could be because cases

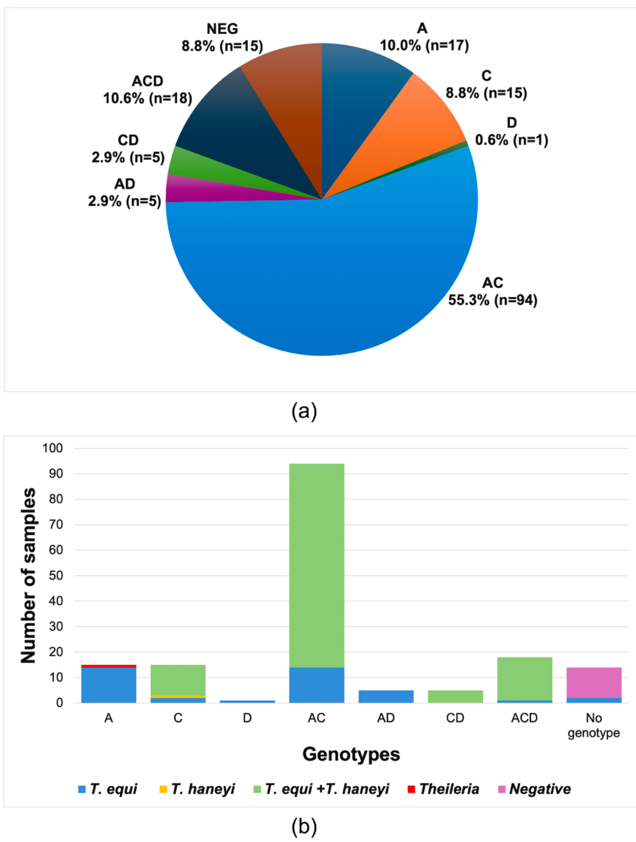

Fig. 7. **(A)** Single and mixed infections of equine *Theileria* parasite genotypes A, B, C, and D as determined by Multiplex EP-AB and Multiplex EP-CDE qPCR assays in 170 field horse samples from three South African provinces. Neg: samples that tested negative for equine *Theileria* parasites using the *Theileria* 18S rRNA gene-specific qPCR included in the Multiplex-EP real-time PCR assay, or samples that could not be genotyped. **(B)** Distribution of single and mixed *T. equi* and *T. haneyi* infections amongst the genotyped field samples, as revealed by qPCR assays that detect *T. equi ema*-1 (Bhoora et al., 2010) and *T. haneyi* (The*ma*-11-specific qPCR assay). The The*ma*-11-specific qPCR assay detected *T. haneyi* infections in samples that were positive for genotype C only, either as a single genotype infection (C) or in combination with other genotypes (AC, CD and ACD). A single *T. haneyi* infection was detected in one genotype C positive sample. *Theileria*: Samples that tested positive for equine *Theileria* parasites using the *Theileria* 18S rRNA gene-specific qPCR included in the Multiplex-EP real-time PCR assay, but did not test positive for either *T. equi* or *T. haneyi*. No genotype: Samples that were negative, or that tested positive for *T. equi ema*-1 but could not be genotyped.

of infections with *B. caballi* usually present with low parasitemia, and animals clear the parasites within four years (de Waal, 1992; Friedhoff and Soule, 1996; Wise et al., 2014). Epidemiological surveys conducted in several other countries also reported either low prevalence or complete absence of *B. caballi* infections in field samples, including Italy, Turkey, Nigeria, Iran, and Paraguay (Ahedor et al., 2023; Grandi et al., 2011; Guven et al., 2017; Kalantari et al., 2022; Sunday Idoko et al., 2020).

Horses screened in this study were predominantly infected with genotypes A and C (90.6 %), either as a single or mixed infection, consistent with results from previous studies investigating genotype diversity (Bhoora et al., 2020; Coultous et al., 2020; Mshelia et al., 2020). Our study reports a single infection of genotype D and mixed infections with genotypes C and D (2.9 %) for the first time in South Africa. The inability to genotype one equine *Theileria* positive sample may be attributed to low parasitemia, as indicated by a high Cq value of 36.7 obtained using the Multiplex-EP assay. However, since this sample also tested positive for *T. equi* using the *T. equi ema*-1 qPCR assay, a new 18S sequence variant not detectable using the genotyping assays could be another explanation. This must be confirmed by sequence analysis of the complete 18S rRNA gene from this sample.

Theileria haneyi was always detected by both the nested PCR assay and the The*ma*-11 qPCR assay in samples that tested positive for genotype C. The 18S sequence of *T. haneyi* is known to group with genotype C 18S sequences in phylogenetic analyses (Knowles et al., 2018). This suggests that genotype C may not be exclusive to *T. equi*, and genotype C positive samples may contain either *T. haneyi, T. equi* or co-infections with both parasites. Additionally, none of the samples that excluded genotype C were positive for *T. haneyi* using the The*ma*-11 qPCR assay, confirming that *T. haneyi* is associated only with genotype C infections in horses in South Africa. The taxonomic relationship between *T. haneyi* and *T. equi* in South Africa can only be confirmed by whole genome sequence analysis of South African genotype C and *T. haneyi* isolates. However, identifying single *T. haneyi* infections in South African horses may be difficult, as only one sample out of the 158 *Theileria*-positive samples examined in this study was positive for *T. haneyi* and not *T. equi*. This sample also tested positive for *B. caballi*, further highlighting the difficulty of identifying single EP infections in horses in South Africa. Additionally, isolating and culturing individual *T. equi* genotypes from South African horses has been challenging, primarily due to the prevalence of co-infections involving multiple parasite genotypes. Given these challenges, isolating *T. haneyi* from horses in South Africa is expected to be particularly difficult.

A qPCR assay targeting a fragment of the gene encoding a hypothetical protein on chromosome 1 (chr1sco gene) of *T. haneyi* was recently developed in China (Zhou et al., 2023). Unlike the The*ma*-11 qPCR assay designed in our study, the Chinese *T. haneyi* qPCR assay was less sensitive than the nested PCR assay in detecting *T. haneyi*. An alignment of the chr1sco gene primer and probe sequences designed by Zhou et al. (2023) with published *T. haneyi* Eagles Pass (MT896770), Egyptian (MW591692-MW591697) and South African (MW591580-MW591586) sequences (Bhoora et al., 2020; Elsawy et al., 2021; Katjivena, 2022; Knowles et al., 2018), indicated 100 % conservation in the primer and probe regions. Based on this observation, the chr1sco qPCR assay for *T. haneyi* should be able to detect *T. haneyi* infections in South African, Egyptian and USA samples. However, not all Chinese samples that tested nested PCR positive for *T. haneyi* were detected by the chr1sco qPCR. It is therefore possible that the Chinese *T. haneyi* chr1sco gene sequences are not as conserved as the published sequences. This highlights the importance of thoroughly assessing sequence variation in the target gene before developing diagnostic assays.

The results obtained using the The*ma*-11 and *T. equi ema*-1 qPCR assays suggest that co-infections of *T. equi* and *T. haneyi* are prevalent in South Africa. South African *T. haneyi ema*-11 sequences are grouped into two distinct phylogenetic clades. To gain a more comprehensive understanding of these infections, it will be essential to determine the correlation between the results obtained from various diagnostic assays. Specifically, future research should compare the outcomes of the *T. equi* EMA-1 cELISA assay (Knowles et al., 1992), the The*ma*-11 indirect ELISA assay (Bastos et al., 2021), the The*ma*-11 qPCR, the *T. equi ema*-1 qPCR (Bhoora et al., 2010), and the *T. haneyi* chr1sco qPCR (Zhou et al., 2023) on South African field samples. This comprehensive analysis will address existing gaps and enhance our understanding of *T. equi* and *T. haneyi* co-infections in the region.

5. Conclusion

The The*ma*-11 qPCR assay developed in our study demonstrated high sensitivity and specificity in detecting *T. haneyi ema*-11, marking a significant improvement over the nested PCR assay, which lacks sensitivity and may miss positive cases. When evaluated alongside the *T. equi ema*-1 qPCR assay, the The*ma*-11 qPCR assay detected *T. haneyi* in 68 % of field samples, while *T. equi* was detected in 92 % of samples. These results suggest that although the prevalence of *T. equi* is much higher in South Africa, there is also a significant presence of *T. haneyi* in the sampled population. Our findings highlight the performance of the Th*ema*-11 qPCR assay in conjunction with the *T. equi ema*-1 qPCR assay, allowing for detection of and differentiation between *T. haneyi ema*-11 and *T. equi ema*-1. Future studies should focus on sequencing the complete *ema* gene complement from South African *T. equi* and *T. haneyi* samples to further elucidate the molecular evolution of these parasites. This comprehensive approach will enhance our understanding of the genetic diversity within these pathogens and improve diagnostic strategies for equine piroplasmosis in South Africa.

Animal health and compliance

Ethical approval was obtained from the University of Pretoria Animal Ethics Committee (AEC) and Research Ethics Committee (REC) (REC111-21). In addition, permission to perform research in terms of Section 20 of the Animal Diseases Act, 1984 (Act No. 35 of 1984) was obtained from the Department of Agriculture, Land Reform and Rural Development (DALRRD) (Act No. 35 of 1984) (12/11/1/1/6 (2156 RJ)).

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CRediT authorship contribution statement

Raksha V. Bhoora: Writing – review & editing, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **Tshenolo V. Mbaba:** Writing – original draft, Methodology, Investigation. **Milana Troskie:** Writing – review & editing, Investigation. **Rebecca E. Ackermann:** Writing – review & editing, Visualization. **Nicola E. Collins:** Writing – review & editing, Supervision, Methodology, Conceptualization.

Declaration of competing interest

The authors 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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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ttbdis.2025.102487.

Data availability

Data will be made available on request.

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