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Genetic diversity in *Babesia bovis* from southern Africa and estimation of *B. bovis* infection levels in cattle using an optimised quantitative PCR assay

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ABSTRACT

Babesia bovis is a causal agent of bovine babesiosis, a disease which leads to mortality and morbidity and impacts the cattle industry worldwide. We amplified, cloned and sequenced the *B. bovis* merozoite surface antigen-2b (*msa-2b*) gene (~940 bp) and the near full-length 18S rRNA gene (~1600 bp) from cattle samples from South Africa and Mozambique to determine sequence variation between *B. bovis* parasites in the region. A TaqMan quantitative real-time PCR (qPCR) assay (18S rRNA gene) was optimised for the detection of *B. bovis* and estimation of parasitaemia in field samples from cattle from southern Africa. Phylogenetic analysis grouped the Msa-2b sequences in six clades and these were 59.7 to 99.6% identical to reference sequences. Sequence variation amongst *B. bovis* 18S rRNA sequences was found at 2 to 36 positions, and the sequences were 97 to 99% identical to published sequences. Mismatches between the *B. bovis* 18S rRNA sequences and a previously published qPCR forward primer (BoF) were observed; therefore, we developed a new forward primer (BoF2), and optimised the qPCR assay. Six 10-fold dilution series of *B. bovis* infected erythrocytes (2×10^8 to 2×10^3 infected red blood cells [iRBC]/ml) were analysed in triplicate in each of six separate qPCR runs, to determine the efficiency of the assay. The qPCR assay amplified the *B. bovis* 18S rRNA gene with 92.0 to 94.9% efficiency. The detection limit of the qPCR assay was approximately 6 iRBCs/µl. The performance of the optimised assay to diagnose *B. bovis* in field samples was assessed by testing DNA from 222 field samples of cattle from South Africa and Mozambique using three methods: the optimised qPCR assay, the reverse line blot (RLB) hybridisation assay, and the previously published qPCR assay. The detection rate of *B. bovis* using the optimised qPCR assay (31.1%, 69/222) was significantly higher ($p<0.001$) than both that using RLB (20.7%, 46/222) and the previously published qPCR assay (5.4%; 12/222). The *B. bovis* parasitaemia in samples from infected cattle ranged from 6 iRBCs/µl to 101,852 iRBCs/µl of blood. Our study revealed marked sequence variation between *B. bovis* parasites from southern Africa. The optimised qPCR assay will be useful in epidemiological studies and clinical diagnosis of *B. bovis* in southern Africa, and can be used to determine parasitaemia and potential carrier status in cattle populations, which is essential in the control of babesiosis.

1. Introduction

Babesia bovis is a tick-transmitted protozoan parasite which infects bovine red blood cells, and is one of two economically important aetiological agents of bovine babesiosis in Africa. While the second pathogen, *Babesia bigemina*, is more widespread, *B. bovis* causes a more virulent form of babesiosis in cattle (Callow, 1979). The disease can cause mortality rates of 70 to 80% in susceptible animals (Zintl et al.,

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2005).

In southern Africa, *B. bovis* has been reported in various areas. In South Africa, 35.5% of cattle tested positive in peri-urban localities in Gauteng Province (Mtshali et al., 2014), and 35.1% in all nine provinces (Mtshali and Mtshali, 2013), using a nested PCR that targets the *B. bovis* rhoptry-associated protein 1 gene (*rap-1*). In another study, using the reverse line blot hybridisation (RLB) assay, Mtshali et al. (2013) showed that the occurrence of *B. bovis* was 1% in the Free State Province of South Africa. The seroprevalence of anti-*B. bovis* antibodies was 39.8% in eight out of the nine provinces of South Africa, using the indirect fluorescent antibody test (IFAT) (Terkawi et al., 2011). In Mozambique, the prevalence of *B. bovis* in Maputo Province was shown to be 53% using a semi-nested hot-start PCR that targets the *B. bovis* putative aspartic proteinase *babesipsin* gene (Martins et al., 2010), while seroprevalence was 78.8% in Maputo, Gaza and Inhambane Provinces, using enzyme-linked immunosorbent assay (ELISA) (Tembue et al., 2011). These reports indicate that bovine babesiosis is endemic in various parts of southern Africa. Therefore, highly sensitive and specific diagnostic methods are needed to monitor the prevalence of *B. bovis*, in order to provide the data needed to implement control strategies. However, difficulties in diagnosing *B. bovis* are likely to occur because parasitaemia is usually low. It is therefore important to detect carriers and sub-clinical infections so as to estimate the level of risk posed by *B. bovis*, in order to inform disease management and control strategies.

Quantitative real-time PCR (qPCR) technologies have been developed and applied to the diagnosis of many organisms including *Babesia caballi* and *Theileria equi* (Lobanov et al., 2018), *Theileria parva* (Sibeko et al., 2008), *Anaplasma marginale* and *Anaplasma centrale* (Chaisi et al., 2017), and *B. bovis* and *B. bigemina* (Kim et al., 2007; Stoltsz et al., 2020). Quantitative PCR assays have several advantages over conventional PCR: qPCR assays are more sensitive, quantification of parasite load is possible, and the risk of contamination is reduced as detection and quantification take place in a single tube during the cycling process, thus eliminating the need for post-PCR manipulation. To our knowledge, qPCR assays have not been applied in the detection and accurate estimation of *B. bovis* parasitaemia amongst cattle raised in *R. microplus*-endemic areas in southern Africa.

An 18S rRNA TaqMan qPCR assay has previously been described for the detection and quantification of *B. bovis*, based on *B. bovis* sequences from Asia (China), Europe (Portugal), North America (Mexico and USA) and the vaccine strains BBOV2 (accession no. L19077) and BBOV3 (L19078) from South Africa, and the test was evaluated using *in vitro* cultured parasites and field blood samples from cattle from Brazil (Kim et al., 2007). However, given the genetic diversity that can occur in pathogens from different localities, it is not known if the assay can effectively detect *B. bovis* from Africa. A high level of genetic diversity has been found amongst South African *B. bovis* field isolates using allele specific primers of the Bv80 gene as markers (Combrink et al., 2014), and genetic variation was demonstrated amongst *rap-1a* sequences from different isolates around the world (Niu et al., 2015). Primers and probes designed in conserved regions of marker genes based on isolates from one region may therefore fail to amplify from divergent lineages in other areas or do so relatively poorly.

Sequence variations of *B. bovis* merozoite surface antigens (MSAs) have been assessed from different regions of the world, including Mongolia (Altangerel et al., 2012), Sri Lanka (Sivakumar et al., 2013), Ghana (Nagano et al., 2013), Israel (Molad et al., 2014), and Brazil (Mendes et al., 2019; Matos et al., 2020). The marked polymorphism of the immunogenic *B. bovis* MSAs, which contain neutralising-sensitive epitopes, enables the parasite to evade the immune system of the host, and results in lack of cross-protection between vaccine strains (Carcy et al., 2006). Therefore, assessment of *B. bovis* MSA sequences can provide vital information for effective recombinant vaccine formulation. There is no data yet on *B. bovis* MSAs from southern Africa.

The objectives of this study were to examine genetic variation in *B. bovis* using *msa-2b* and 18S rRNA sequences from field samples from cattle in southern Africa, and to apply an optimised 18S rRNA TaqMan-based qPCR assay to detect *B. bovis* positive cattle blood samples and determine the infection levels.

2. Materials and methods

2.1. Ethics statement

The study was approved by the Animal Ethics Committee of the University of Pretoria, South Africa (V047-12). Permission was obtained to do research in terms of Section 20 of the Animal Diseases Act, 1984 (Department of Agriculture, Land Reform and Rural Development - Pretoria, South Africa; reference number 12/11/1/1/6). Standard techniques were followed in collecting blood samples for laboratory examination.

2.2. Field samples from cattle

Cattle blood samples were collected in vacuum tubes coated with ethylenediaminetetraacetic acid (EDTA); the samples were from studies conducted at the Department of Veterinary Tropical Diseases (DVTD), University of Pretoria from 2015 to 2018 (Choopa, 2015; Nyoni-Phili, 2017; Makgabo, 2018). The sampling areas were the Mnisi community in Mpumalanga Province, South Africa (n=122) and Namitangurine and Botao villages located in the Zambezia Province, Mozambique (n=100). All available Mozambican samples were included in this study, while South African specimens were selected by systematic random sampling, in which a sampling interval was calculated by dividing the total number of available specimens by the required number of samples. DNA was extracted from the blood samples using a QIAamp® DNA Mini Kit (QIAGEN, Hilden, Germany) following the manufacturer's protocol.

2.3. Reverse line blot (RLB) hybridisation assay

A total of 222 field samples were tested using the RLB hybridisation assay as previously described (Nijhof et al., 2003; 2005). The V4 hypervariable region of the 18S rRNA gene for *Theileria/Babesia* species was amplified (Nijhof et al., 2003; 2005) using Platinum® Quantitative PCR SuperMix-UDG (Invitrogen, The Scientific Group, South Africa). DNA extracted from *B. bovis* live blood vaccine (Onderstepoort Biological Products [OBP], Pretoria, South Africa) was used as the positive control for the 18S rRNA PCR. PCR grade water was included in the negative control reaction instead of template DNA.

2.4. Babesia bovis msa-2b and 18S rRNA sequence analyses

Babesia bovis msa-2b sequences were obtained by amplifying the ~940-bp gene from samples that displayed a positive signal with the *B. bovis*-specific RLB probe, as well as from the South African *B. bovis* vaccine strain (OBP, Pretoria, South Africa). The 18S rRNA sequences were obtained by amplifying the near full-length gene (~1600 bp) from 37 samples that showed a positive signal with *Babesia* genus-specific probes (*Babesia* catch-all 1 and/or *Babesia* catch-all 2), with or without *B. bovis*-specific signal during RLB analysis.

The *B. bovis msa-2b* gene was amplified using primers MSA-2b F (5'-ATG ATC GGG AAA ATC TTC TTG TTA A -3') and MSA-R (5'- TTA AAA TGC AGA GAG AAC GAA GTA GC -3') (Sivakumar et al., 2013). The PCR conditions were as previously described (Sivakumar et al., 2013), with some modifications according to the Phusion™ Flash High-Fidelity PCR Master Mix protocol (Thermo Scientific™, Waltham, Massachusetts, United States). Each reaction mixture contained 1X Master Mix, 1 μM of each primer, 2.0 μl of template DNA and nuclease-free water to a total volume of 25 μl. The amplification cycles comprised a denaturation stage at 98°C for 10 s, followed by 40 cycles at 98°C for 30 s, 52°C for 1 min and 72°C for 2 min, and a final extension at 72°C for 7 min. The *B. bovis* 18S rRNA gene was amplified using primers EK-1F (5'- CTG GTT

GAT CCT GCC AG -3') (Lefranc et al., 2005) and 18S-1498R (5'-CAC CTA CGG AAA CCT TGT TA -3') (López-García et al., 2003). Optimal PCR conditions were determined in our laboratory following the Phusion™ Flash High-Fidelity PCR Master Mix protocol (Thermo Scientific™, Waltham, Massachusetts, United States). The reaction mixtures contained 1X Master Mix, 0.2 μM of each primer, 2.5 μl of template DNA and nuclease-free water to a total volume of 25 μl. The amplification cycles comprised a denaturation stage at 98°C for 30 s, followed by 35 cycles at 98°C for 10 s, 60°C for 30 s and 72°C for 30 s, and a final extension at 72°C for 10 min. In both PCRs, PCR grade water was included in the negative control reaction, while DNA extracted from the *B. bovis* live blood vaccine (OBP, Pretoria, South Africa) was included as a positive control. The PCR products were analysed by electrophoresis on 2% TAE agarose gels stained with ethidium bromide.

The PCR products were extracted from the gel using the QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany) or purified using the QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany), followed by cloning using the CloneJET® PCR Cloning Kit (Thermo Scientific™, LTC Tech South Africa [Pty] Ltd, Randburg, South Africa) and JM109 competent cells (Promega, Madison, USA). Recombinant plasmid clones were screened by colony PCR as previously described (Byaruhanga et al., 2018). The amplicons generated from recombinant clones were sequenced at the Inqaba Biotec sequencing facility (Inqaba Biotechnical Industries [Pty] Ltd, Pretoria, South Africa) using primers pJET1.2F (5'-CGA CTC ACT ATA GGG AGA GCG GC-3') and pJET1.2R (5'-GAA GAA CAT CGA TTT TCC ATG GCA G-3'). The *msa-2b* and 18S rRNA sequences obtained in this study were aligned with worldwide *B. bovis* *msa-2b* and 18S rRNA sequences from GenBank using Multiple Alignment with the Fast Fourier Transform (MAFFT) version 7.0 (Katoh and Standley, 2013). The *msa-2b* nucleotide sequences were translated to amino acids using the EMBOSS Transeq program (https://www.ebi.ac.uk/Tools/st/emboss_transeq/), and percent similarity and identity amongst the sequences were calculated using the Sequence Identity And Similarity (SIAS) tool (http://imed.med.ucm.es/Tools/sias.html). The evolutionary divergence (number of nucleotide differences and percentage identity) between various *B. bovis* sequences was estimated over a length of 1,592 bp of the 18S rRNA gene using the Molecular Evolutionary Genetics Analysis version 7.0 (MEGA7) (Kumar et al., 2016).

The 14 *B. bovis msa-2b* sequences and the 13 unique 18S rRNA sequences obtained in this study were deposited in GenBank under accession numbers **MT720706** to **MT720717, OM240817 to OM240819 and OL361842 (*msa-2b*),** and **MH257724** to **MH257736 (18S rRNA)**.

2.5. Babesia bovis Msa-2b phylogenetic analysis

The best-fit evolutionary model, JTT + G + F, for the Msa-2b amino acid sequences was selected using the ProtTest 3 program (Darriba et al., 2011), based on the Akaike Information Criterion (AIC). A maximum likelihood phylogenetic tree was constructed with the newly identified and reference Msa-2b amino acid sequences using the PhyML program (version 3.1) (Guindon et al., 2010). Bootstrap analysis was done with 1000 replicates to estimate the confidence levels of the tree branches (Felsentein, 1985).

2.6. qPCR analyses

2.6.1. Initial testing of field samples with the published B. bovis qPCR assay

A previously published *B. bovis* qPCR assay (Kim et al., 2007) designed using sequences obtained from China, Portugal, Mexico and the USA, and evaluated using field blood samples from cattle from Brazil, was used to test the DNA extracted from 222 field samples from cattle from South Africa and Mozambique. Primers BoF (5'-AGC AGG TTT CGC CTG TAT AAT G-3') and BoR (5'-AGT CGT GCG TCA TCG ACA AA-3') and probe BoP (5'-6-FAM-CCT TGT ATG ACC CTG TCG TAC CGT TGG-TAMRA-3') (Kim et al., 2007), and the qPCR conditions previously described (Kim et al., 2007) were used in a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Life Technologies, Johannesburg, South Africa).

2.6.2. Optimisation of qPCR conditions

Based on *B. bovis* 18S rRNA sequences obtained in this study, a new forward primer, BoF2 (5'-GGT TTC GCC TGT ATA ATT G-3'), was designed for the optimised qPCR assay. The qPCR assay was tested with different annealing temperatures (52, 54, 55 and 57°C), primer concentrations (0.2, 0.5 and 0.8 μM), probe concentrations (0.1, 0.15, 0.25 and 0.4 μM) and Master Mix concentrations (0.8X, 1X and 1.2X) in order to find the optimal conditions that resulted in lowest quantification cycle (C_q) values, least standard deviation (SD) between C_q values, and with robust fluorescence curves parallel to each other amongst replicates. Optimisation was performed using five *B. bovis* positive samples, confirmed as such by sequence analysis of the 18S rRNA gene. Each sample was tested in triplicate.

2.6.3. Optimal qPCR conditions

Once the qPCR assay was optimised, primers BoF2 (this study) and BoR (Kim et al., 2007), and probe BoP (Kim et al., 2007) were used to amplify and detect a 153 bp fragment of the *B. bovis* 18S rRNA gene. Each PCR reaction comprised 0.8X TaqMan® Universal PCR Master Mix (Applied Biosystems, Life Technologies, Johannesburg, South Africa), 0.5 μM of each oligonucleotide primer, 0.25 μM of the VIC and TAMRA-labelled probe and 2 μl of DNA template in a total reaction volume of 20 μl. Thermal cycling was done in a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Life Technologies, Johannesburg, South Africa) under the following conditions: Uracil N-Glycosylase digest at 50°C for 2 min, followed by AmpliTaq Gold pre-activation at 95°C for 10 min and then 45 cycles of amplification at 95°C for 20 s and annealing at 57°C for 1 min. Positive and negative controls were as in Section 2.4 above.

2.6.4. Standard curves and efficiency of the optimised qPCR

Babesia bovis-infected erythrocytes (2×10^8 infected red blood cells [iRBC]/ml of blood) from the live blood vaccine (OBP, Pretoria, South Africa) were used to generate standard curves to determine the efficiency of the qPCR. Six 10-fold dilution series (2×10^8 to 2×10^3 iRBC/ml of blood) of infected erythrocytes in uninfected bovine blood as the diluent (confirmed to be *B. bovis* negative using RLB hybridisation assay and qPCR) were prepared in triplicate, and genomic DNA was extracted from 200 μl of each dilution using the QIAamp® DNA Mini Kit (QIAGEN, Hilden, Germany). Each dilution was tested in triplicate in each of six qPCR assay runs. The average of the C_q values from the dilutions in each qPCR test run were plotted against the logarithm of the parasitaemia (log [iRBCs/qPCR reaction]) to generate six standard curves. Each qPCR run contained negative and positive controls as indicated in Section 2.4. The PCR efficiency [E] (Pfaffl, 2001; Vandesompele et al., 2002), expressed as a percentage, was determined using the following formula:

$$\%E = \left(10^{(-1/\text{Slope})} - 1\right) x100$$

where slope = slope of the derivative (tangent line) of the calibration curve.

2.6.5. Analytical specificity of the qPCR assay

The analytical specificity of the assay was assessed by testing DNA extracted from other protozoal parasites, namely *Theileria parva* (from blood of a naturally infected African buffalo, *Syncerus caffer*, sample KNP102 from the Kruger National Park, South Africa), *B. bigemina* (live blood vaccine, OBP, Pretoria, South Africa), *B. occultans* (diagnostic bovine blood sample RE18/016 from the Molecular Diagnostic Laboratories of the DVTD, University of Pretoria, South Africa), *B. microti* (DNA kindly provided by Frans Jongejan, Utrecht University, Utrecht, the Netherlands), *B. rossi* (diagnostic dog blood sample RE16/016 from the

Onderstepoort Veterinary Academic Hospital [OVAH], Pretoria), *B. vogeli* (diagnostic dog blood sample RE15/253 from OVAH), *B. caballi* (tissue culture obtained from Onderstepoort Veterinary Institute as a diagnostic sample) *T. taurotragi, T. mutans, T. velifera* (diagnostic bovine blood sample RLB 18/008 from DVTD) and *T. equi* (diagnostic equine blood sample RLB 12/058 from DVTD). A negative control, prepared by extracting DNA from blood from a piroplasm-free bovine (confirmed using RLB hybridisation assay, indirect fluorescent antibody test and qPCR), was included in each assay.

2.6.6. Limit of detection (analytical sensitivity)

Eight 2-fold dilution series were prepared using the 2×10^3 iRBC/ml dilution of *B. bovis* infected RBCs reported above (Section 2.6.4), to obtain parasite concentrations from 1,000 to 7.81 iRBC/ml. The diluent was blood from a *B. bovis*-negative bovine (confirmed using RLB hybridisation assay, indirect fluorescent antibody test and qPCR). DNA was extracted from five aliquots of each dilution using the QIAamp® DNA Mini Kit (QIAGEN, Hilden, Germany) and tested in five independent qPCR runs (total 25 replicates per dilution). DNA from the original *B. bovis* live blood vaccine and from uninfected bovine blood were included in each run as positive and negative controls, respectively. The results of these analyses were used to calculate the limit of detection (LOD) of the *B. bovis* DNA, using probit analysis in the Statistical Package for the Social Sciences (SPSS) software version 25.0 (IBM SPSS, 2017). The LOD was the input concentration giving a *B. bovis* qPCR positive test result in 95% of the replicates (Burns and Valdivia, 2008). Positives were defined as those that showed amplification in the amplification plot and had C_q values of <39.

2.7. Detection and quantification of B. bovis in field samples, and comparison with other methods

The optimised assay was used to establish the occurrence and estimated parasitaemia of *B. bovis* in 222 DNA samples from cattle from South Africa and Mozambique. Each sample was tested in duplicate. The standard curves generated previously (Section 2.6.4) were used to estimate *B. bovis* parasitaemia (iRBC/µl of blood) in the field samples, using linear regression. If the standard deviation of the C_q between duplicates of a sample was greater than 0.8, the sample was tested again.

The results were compared with *B. bovis* detection using RLB and the previously published qPCR assay (Kim et al., 2007).

2.8. Statistical analysis

The Chi-square test was performed to determine significant differences in detection rates of *B. bovis* between the three methods: the RLB hybridisation assay, the previously published qPCR and the optimised qPCR. The independent samples *t*-test was performed to compare the C_q values from the previously published assay and the optimised qPCR assay. Cohen's kappa (*k*) test was used to determine the level of agreement (Landis and Koch, 1977) between the test methods for the detection of *B. bovis*. The data were analysed using SPSS version 25.0 (IBM SPSS, 2017) at 5% level of significance.

3. Results

3.1. Reverse line blot (RLB) hybridisation assay

Of the 222 field samples (100 from Mozambique, 122 from South Africa) that were tested for *Theileria, Babesia, Anaplasma* and *Ehrlichia* species using the RLB hybridisation assay, 113 samples (50.9%) gave positive signals with *Babesia* genus-specific probes, with or without *Babesia* species-specific probe signals. *Babesia bovis* was detected in 46 samples (20.7%), of which 33 were from South Africa (27%, 33/122) and 13 samples were from Mozambique (13%, 13/100).

3.2. Babesia bovis Msa-2b sequence and phylogenetic analysis

The *msa-2b* gene was successfully amplified from 10 field samples that tested positive for *B. bovis* (six from South Africa and four from Mozambique) and from the South African *B. bovis* vaccine strain. The PCR products were cloned and two to three clones from each sample were sequenced, first using the forward primer pJET1.2F for all recombinant clone amplicons and then pJET1.2R for only amplicons that corresponded to *B. bovis msa-2b* gene on Basic Local Alignment Search Tool (BLAST) search (n=4 from Mozambique and n=9 from South Africa). Other clone sequences obtained using the pJET1.2F primer corresponded to 'uncultured bacteria', had insignificant similarity to sequences in GenBank, or matched with *B. bovis msa-2a1* and *msa-2a2* genes– and therefore their amplicons were not sequenced with the reverse primer pJET1.2R. A total of 14 *B. bovis msa-2b* sequences (781 to 933 bp) were identified: four from the four samples from Mozambique, nine from the six samples from South Africa and one from the South African vaccine strain (788 bp).

Analysis of the *B. bovis* Msa-2b amino acid sequences (260 to 311 amino acid residues) showed significant variation, even between sequences from the same sample. Sequence similarity ranged from 55 to 100% (62.3 to 100% identity) among South African samples and from 57 to 100% (65.0 to 100% identity) among samples from Mozambique. The sequences had 65.7 to 99.6% similarity (59.9 to 99.6% identity) to a sequence from cattle from Philippines (GenBank Accession no. AB819784) and 69.0 to 87.5% similarity (61.1 to 84.7% identity) to the Australian breakthrough field isolate G06 (DQ173950) from cattle vaccinated with the Australian T vaccine strain (DQ173958). There was 67.1 to 81.2% similarity (60.1 to 74.2% identity) to the Israel vaccine strain sequence (KJ144250) and 67.3 to 86.5% similarity (59.7 to 83.3% identity) to the Australian virulent strain L (DQ173955). The *B. bovis* Msa-2b sequences from field samples had 65.5 to 98.1% similarity (59.0 to 97.3% identity) to the South African vaccine strain sequence (OL361842).

Phylogenetic analysis showed that the newly-identified *B. bovis* Msa-2b sequences grouped in six clusters, together with reference sequences from various countries in four continents: Asia, North America, South America and Australia (Fig. 1). The Msa-2b sequences from Mozambique grouped in two different clusters, while the South African Msa-2b sequences grouped in six clusters (Fig. 1). None of the sequences obtained in this study grouped with the sequence from the Israel vaccine strain (Clade 5), but one sequence from Mozambique and one sequence from South Africa grouped with the Australian vaccine strain T (clade 9) and three sequences each from Mozambique and South Africa grouped with the South African vaccine strain sequence in clade 7 (Fig. 1).

3.3. Babesia bovis 18S rRNA sequences

Six to ten clones were sequenced from each 18S rRNA amplicon. Thirteen unique *B. bovis* 18S rRNA gene sequences (1447 bp to 1636 bp) were identified from 16 clones from three out of 37 field samples that gave a positive signal with *Babesia* genus-specific probes (*Babesia* catch-all 1 and/or *Babesia* catch-all 2). The other 34 samples did not yield any *B. bovis* sequences. Sequence analysis showed diversity amongst the *B. bovis* 18S rRNA sequences obtained (variation 2 to 36 nucleotides), and these differed from published sequences in GenBank by 2 to 61 nucleotides (S1 Table). The new sequences had 97% identity to those from the USA (GenBank accession number: M87566) and Portugal (AY150059), 98% identity to a sequence from Australia (JQ437262), and 99% identity to those from China (JQ723013), India (KF928959), Mexico (L31922), Brazil (EF458213) and the South African vaccine strain (L19077) [query cover 97 to 100%, over a length of 1592 bp]. Sequences of related haemoparasites were obtained from South African samples, and these correspond to *B. bigemina* (nine samples), *Theileria velifera* (seven samples), *Theileria* sp. strain MSD (three samples), and *Theileria taurotragi* (two samples). From one sample, sequences

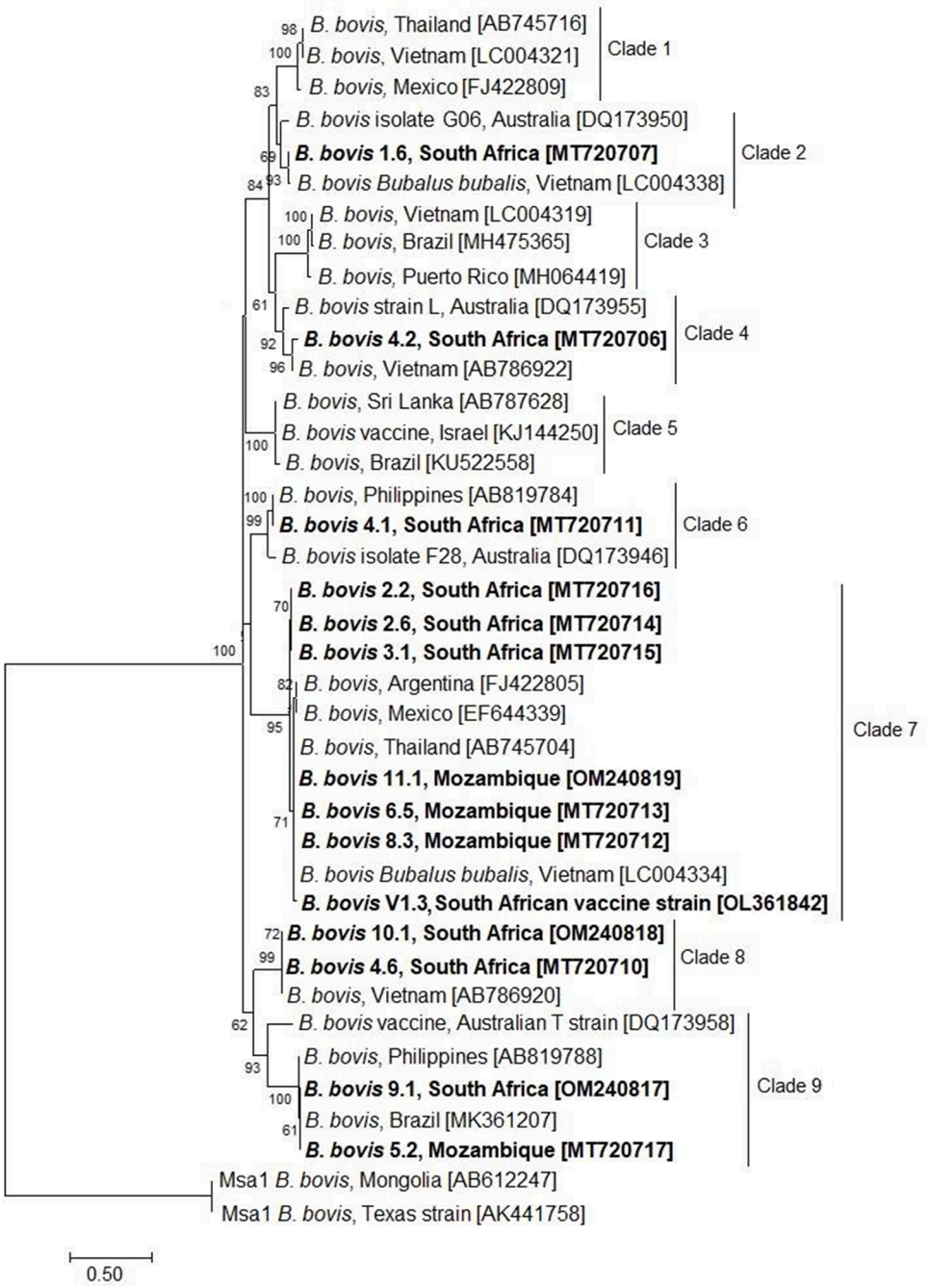

Fig. 1. Phylogenetic analysis of *Babesia bovis* Msa-2b amino acid sequences identified in blood samples from cattle from South Africa and Mozambique (in bold) with reference sequences. The phylogenetic tree was constructed using the maximum likelihood method. The numbers at the internal nodes represent the percentage of 1000 replicates (bootstrap) for which the same branching patterns were obtained. The tree was rooted using *B. bovis* Msa-1 amino acid sequences from a Texas strain and from a Mongolian bovine sample. The accession number of each sequence is indicated next to the sequence name. Sequences with the same numerical digit in front of the point originate from the same cattle sample; the numerical digit following the point indicates the clone number. Branch lengths are proportional to the estimated genetic distance (number of amino acid substitutions per site over a length of 369 positions of the Msa-2b amino acid sequences) between the taxa.

corresponding to wild canine apicomplexan parasites, *Hepatozoon felis* and *Babesia lengau*, were identified (99 to 100% identity to GenBank accessions).

When the qPCR primer and probe positions were examined, the probe (BoP) and reverse primer (BoR) sequences (Kim et al., 2007) were identical to the target regions in the *B. bovis* sequences. However, there was a mismatch at the 3' end of the target sequence of the forward primer (BoF) in the *B. bovis* sequences obtained in the present study and those from other continents, with a TG at the 3' end of the primer sequence and a TTG in the *B. bovis* 18S rRNA sequences (S1 Figure). The 3' position is crucial for binding of primers to templates, and a single mismatch in this position is sufficient to prevent specific amplification, suggesting that the previously published qPCR assay (Kim et al., 2007) would not detect all *B. bovis* samples. However, we suspect that there was a typographical error in the BoF sequence in the original publication, since all of the *B. bovis* sequences that were used by Kim et al. (2007) to design the primer, matched the sequences that we obtained from southern African *B. bovis* samples (S1 Figure).

3.4. Initial testing of field samples with the published B. bovis qPCR assay

It was found that out of 46 samples that displayed positive signals with RLB, 37 were negative (quantification cycle [C_q]>39) when tested using the previously published qPCR assay (Kim et al., 2007) (Table 1).

3.5. Optimisation of the qPCR assay

We designed a new forward primer, BoF2 (5'-GGT TTC GCC TGT ATA ATT G-3'), for the qPCR assay (S1 Figure). Optimum amplification and quantification of *B. bovis* DNA in the qPCR assay was observed at an

Table 1
Comparison of detection rates of *Babesia bovis* DNA from 222 cattle blood samples using the reverse line blot (RLB) hybridisation assay and the previously published quantitative real-time PCR (qPCR) assay (Kim et al., 2007).

	Previously published qPCR		
RLB	Positive	Negative	Total
Positive	9 (4.1)[a]	37 (16.7)	46 (20.7)
Negative	3 (1.4)	173 (77.9)	176 (79.3)
Total	12 (5.4)	210 (94.6)	222

[a] Figures in parenthesis represent the percentage of positive or negative samples.

annealing temperature of 57°C and a Master Mix concentration of 0.8X. From these conditions, the lowest C_q values, with an average SD of 0.45, and more robust levels of fluorescence were obtained.

3.6. Standard curves, efficiency and quantification of parasitaemia in field samples

Linear regression analysis from the six qPCR assays showed that the optimised assay had a linear range over six logs, from 2×10^8 to 2×10^3 iRBC/ml of blood containing *B. bovis*-infected erythrocytes (S2 Fig. and Table 2). The assay was 92.0 to 94.9% efficient in the amplification of a 153 bp fragment of the 18S rRNA gene (Table 2). A representative standard curve from the serial dilutions is shown in S2 Figure. The correlation coefficients (R^2) and slopes were close to the theoretical optimums, showing that the tests precisely quantified the DNA from *B. bovis* within the limits considered.

3.7. Analytical specificity of the B. bovis qPCR assay

No amplification was observed from negative control samples from donor cattle (free of infection) or DNA extracted from other protozoal parasites, demonstrating the specificity of the assay. Sequence analysis showed sufficient mismatches between sequences of other protozoal parasites and the qPCR primers and probes (data not shown), therefore indicating no likelihood for erroneous detection of other haemoparasites by the *B. bovis* qPCR assay.

3.8. Limit of detection/analytical sensitivity of the B. bovis qPCR assay

When the reliability of the qPCR assay to detect a range of parasitaemias was assessed by testing 2-fold dilution series of infected erythrocytes, *B. bovis* DNA was detected up to the fourth 2-fold dilution containing 125 iRBC/ml of blood. The limit of detection (i.e. input concentration giving a positive result in 95% of the replicates) of *B. bovis* by the qPCR was determined by probit analysis to be 12 iRBC per qPCR reaction or 6 iRBC/µl (Fig. 2), an equivalent of 1×10^{-4}% parasitised erythrocytes, assuming 6×10^6 RBC/µl of blood in bovines (Cornell University, 2014). This corresponds to a C_q of 38.5.

Table 2
Performance of the quantitative real-time PCR assay optimised for the detection and quantification of *Babesia bovis* parasitaemia from cattle in southern Africa.

Assay[a]	Efficiency (%)	Coefficient of determination (R^2)	Slope	y-intercept
1	92.93	0.9933	-3.5036	42.49
2	92.18	0.9939	-3.5247	42.53
3	94.85	0.9936	-3.4518	42.23
4	94.33	0.9924	-3.4657	42.37
5	93.80	0.9984	-3.4799	40.99
6	92.00	0.9899	-3.5298	42.49
Mean ± SD	93.35 ± 1.16	0.9936 ± 0.0028	-3.4926 ± 0.032	42.18 ± 0.595

[a] Standard curves were generated from six separate assays. In each assay, the mean of quantification cycles (C_q) from three replicates was plotted against the logarithm of the input 10-fold dilution series of blood containing 2×10^8 to 2×10^3 infected red blood cells per ml of blood (or 2.6×10^6 to 2.6×10^1 iRBC/qPCR reaction). The 10-fold dilutions were obtained by spiking *B. bovis* live blood vaccine of known parasite concentration in non-infected bovine blood.

3.9. Detection of B. bovis DNA from field samples: comparison between different methods

Of the 122 samples from South Africa tested by the optimised qPCR, 54 (44.3%) were positive for *B. bovis*, while the pathogen was detected in 15% (15/100) of samples from Mozambique. The range of *B. bovis* parasitaemia in the field samples of infected cattle was 6 iRBC/µl to 101,852 iRBC/µl (C_q range of 38.5 to 23.9) of blood. This equates to 1.0×10^{-4}% to 1.7×10^{0}% parasitised erythrocytes (PE). There was no amplification in the negative controls.

Table 3 shows the results from testing 222 field blood samples for *B. bovis* DNA from cattle from South Africa and Mozambique using the three methods: the RLB hybridisation assay, the previously published qPCR (Kim et al., 2007) and the optimised qPCR. The detection rate using the optimised qPCR assay was significantly higher ($p<0.001$) than the detection rate using RLBand the previously published qPCR assay (Table 3). There was substantial agreement between RLB and the optimised qPCR ($k=0.69$, $p<0.001$), but only fair agreement between RLB and the previously published qPCR ($k=0.22$, $p<0.001$) and between the previously published qPCR and the optimised qPCR ($k=0.23$, $p<0.001$), in testing for *B. bovis* in field samples. The mean C_q (± SD) from the positive field samples using the optimised qPCR was significantly lower (29.4 ± 3.9) than that of the previously published qPCR assay (35.0 ± 4.0) ($p=0.004$).

4. Discussion

We determined sequence variation in the *B. bovis msa-2b* and 18S rRNA genes obtained from field sampled cattle in South Africa and Mozambique, and showed that a mismatch at the 3' end of the forward primer in *B. bovis* 18S rRNA gene sequences reduced the accuracy of a previously published qPCR assay. We described the application of an optimised TaqMan qPCR assay for the detection and estimation of *B. bovis* infection levels in cattle from southern Africa.

There was variation amongst the *B. bovis* Msa-2b and 18S rRNA sequences obtained in this study, and these differed from published sequences. The observed Msa-2b variation is consistent with previous studies in Mongolia (Altangerel et al., 2012), Brazil (Mendes et al., 2019; Matos et al., 2020), Israel (Molad et al., 2014) and Sri Lanka (Sivakumar et al., 2013), in which remarkable variation of the MSAs was observed. The grouping of seven of the southern African sequences in one cluster (clade 7), suggests the presence of a relatively predominant *B. bovis msa-2b* allele. The newly-identified *B. bovis* Msa-2b sequences from southern Africa were divergent from the Israel vaccine strain (KJ144250) (Clade 5), and grouped with (n=6) or were divergent from (n=7) the South African vaccine strain, which may suggest that the vaccine may not cross-protect against all field strains. However, although Bv80 gene analysis of the South African *B. bovis* S24 vaccine strain revealed only one Bv80 genotype population (allele A) (Combrink et al., 2014), the vaccine was able to protect cattle against challenge from five out of six local field isolates containing single or multiple parasite populations (Troskie et al., 2017). Protection against the remaining isolate was inadequate, but this was associated with only minor mortalities in vaccinated cattle compared to the mortalities encountered in unvaccinated herds (Troskie et al., 2017). The six isolates sampled for the challenge possessed 13 unique Bv80 genotypes and were from six locations in three provinces (Mpumalanga, KwaZulu-Natal and Eastern Cape) (Troskie et al., 2017). Nevertheless, Berens et al. (2005) found that the Msa-2a/b proteins in all but one of eight Australian *B. bovis* breakthrough isolates examined were markedly different from the Msa-2a/b proteins of the Australian vaccine strains

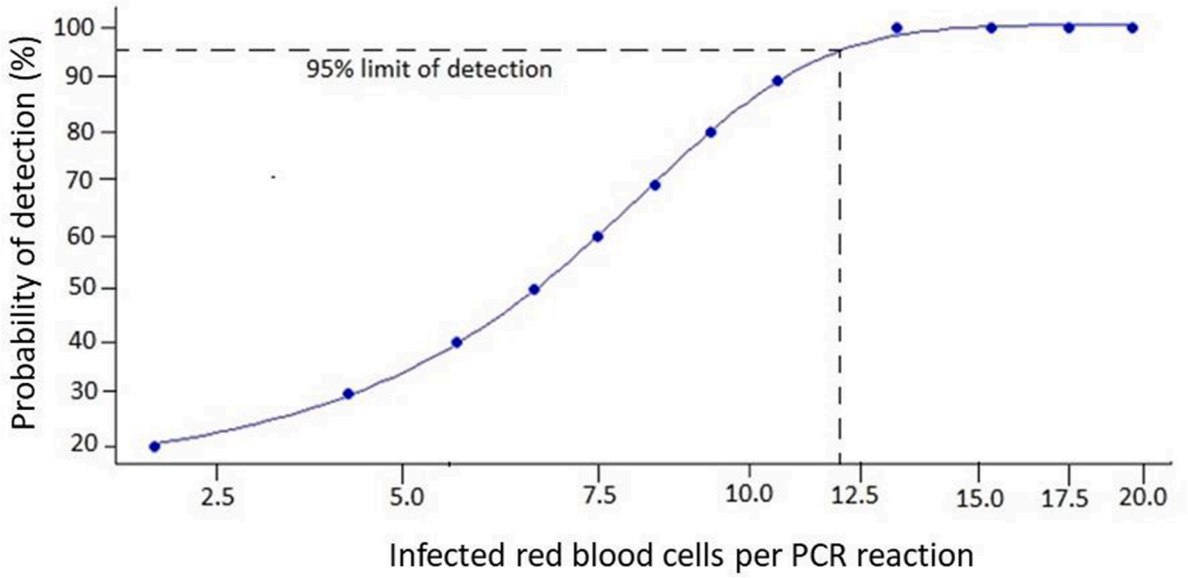

Fig. 2. Probit analysis of two-fold dilution series prepared from a 10-fold dilution of *Babesia bovis* infected erythrocytes in bovine blood free from piroplasms. The points on the sigmoid curve represent the detection probability derived from 25 replicates (five independent runs with five replicates per run) for each serial dilution. The dashed lines on the sigmoid curve signify the 95% limit of detection: approximately 12 infected erythrocytes per real-time PCR reaction, corresponding to 6 iRBCs/µl of blood.

Table 3
Comparison of detection rates of *Babesia bovis* DNA from 222 cattle blood samples using three methods: the optimised 18S rRNA quantitative real-time PCR (qPCR) assay, the reverse line blot (RLB) hybridisation assay and the previously published qPCR assay (Kim et al., 2007).

	RLB		Previously published qPCR		
Optimised qPCR	Positive	Negative	Positive	Negative	Total
Positive	46 (20.7)[a]	23 (10.4)	12 (5.4)	57 (25.7)	69 (31.1)
Negative	0 (0)	153 (68.9)	0 (0)	153 (68.9)	153 (68.9)
Total	46 (20.7)	176 (79.3)	12 (5.4)	210 (94.6)	222

[a] Figures in parenthesis represent the percentage of positive or negative samples.

that had been used to immunise the animals. Given the sequence diversity observed in the present study, further investigation is needed regarding efficacy of the South African vaccine against diverse field strains.

The variation observed in the southern African 18S rRNA gene sequences is consistent with the genetic divergence for *B. bovis* reported previously (Calder et al., 1996). Variation between *B. bovis* 18S rRNA genes can be attributed to strain differences, or to sequence heterogeneity between the three copies of the 18S rRNA gene within individual *B. bovis* genomes (Brayton et al., 2007). However, the identification of 13 unique *B. bovis* 18S rRNA gene sequences from just three samples suggests that these samples were infected with multiple strains of the parasite.

Hepatozoon felis (Baneth et al., 2013) and *B. lengau* (Bosman et al., 2010) 18S rRNA sequences were identified from one sample from a bovine from the Rolle area of the Mnisi community. These are pathogens of the domestic cat (*Felis catus*) and cheetah (*Acinonyx jubatus*) respectively, and are likely to be accidental infections in cattle. Detection in cattle could be attributed to the close proximity of the grazing areas to the Kruger National Park, with the possibility that adult ticks that fed on wild animals in their larval and nymphal stages could transmit the pathogens to domestic animals. Tick species that have been found to feed on wild felids and domestic cats in southern Africa (South Africa, Namibia, Botswana), including *Amblyomma hebraeum* (cheetahs, lions [*Panthera leo*], leopards [*Panthera pardus*], domestic cats), *Rhipicephalus simus* (cheetahs, lions, domestic cats), *Amblyomma marmoreum* (caracals [*Caracal caracal*]), *Rhipicephalus evertsi evertsi* (caracals, leopards), *Hyalomma truncatum* (lions), *Rhipicephalus turanicus* (lions, domestic cats) and *Rhipicephalus zambesiensis* (leopards, domestic cats) (Horak et al., 2010), are known ectoparasites of cattle (Walker et al., 2013).

Given its efficiency, the optimised TaqMan qPCR assay for *B. bovis* can be used for quantitative estimation of parasitaemia in clinical and carrier samples. The assay was highly sensitive and was able to detect as few as 0.0001% parasitised erythrocytes. This is similar to the detection limit reported for the qPCR developed for *B. caballi* (0.000114% PE) in equids from South Africa (Bhoora et al., 2010) and that of *T. parva* (0.000879% PE) from cattle and African buffalo (*Syncerus caffer*) from South Africa (Sibeko et al., 2008). The assay was specific for *B. bovis*, with the primers and probe only amplifying and detecting *B. bovis* DNA and not any of the other protozoal parasites tested.

There was generally low parasitaemia in the field samples from cattle from both South Africa and Mozambique, indicating a carrier status in the cattle population. Generally low numbers of *B. bovis* parasites occur in peripheral blood (Fahrimal et al., 1992), and, while the parasitaemia is between 0.1 and 0.3% in acutely infected cattle (Shkap et al., 2005), it is usually below 10^{-8} in carrier or persistently infected animals (Calder et al., 1996). This is due to sequestration of erythrocytes by attachment to the capillary endothelium (Bock et al., 2004). The optimised qPCR assay therefore provides a useful tool for detection of *B. bovis* even at extremely low parasitaemias that may escape detection by other diagnostic methods, and which could present the risk of transmission among cattle.

A significantly higher detection rate of *B. bovis* in southern African field samples was observed using the optimised qPCR than RLB and the previously published qPCR. The presence of a mismatch at the 3' end of the forward primer (TG in the primer sequence and a TTG in *B. bovis* sequences) could have affected primer annealing to the *B. bovis* template in some samples, leading to lower sensitivity of the previously published qPCR. Stronger binding of G and C bases promotes specific binding at the 3' end, and the extension of the 3' end of annealed primers by the 5'-3'

polymerase activity of the DNA polymerase will not occur unless the 3' end of the oligonucleotide forms a double strand with the template. For RLB, the lower detection rate can be explained by competition for reagents in the PCR reaction, due to simultaneous amplification and detection of the 18S rRNA genes (460 to 520 bp) of various *Theileria* and *Babesia* spp.

About half (44.3%) of the sampled cattle from South Africa were positive for *B. bovis*. The moderately high occurrence of *B. bovis* and the presence of the vector tick, *R. microplus*, throughout the year, may lead to an adequate infection rate of haemoparasites to ensure that young animals are infected while they are protected by innate and/or colostral immunity. Subsequently, constant reinfection maintains parasitaemia at subclinical levels, guaranteeing lifetime immunity (Mahoney and Ross, 1972), a situation called endemic stability. Such animals will however serve as reservoirs for further *B. bovis* transmission. Moreover, outbreaks of babesiosis and losses in the cattle industry may still occur in areas that have previously attained endemic stability, as in situations of inadequate tick control (Sserugga et al., 2003), lack of cross-protection between genetically diverse *B. bovis* strains (Shkap et al., 1994) or due to antigenic variation (Mahoney and Goodger, 1969 cited in Bock et al., 2004). Clinical cases can also occur in calves between the age of 30 and 120 days, a period that coincides with a significant fall in the levels of colostral antibodies (Mahoney and Ross, 1972).

In this study, the proportion of *B. bovis* positive cattle (44.3%) in the Mnisi community, Mpumalanga, South Africa was found to be higher than that reported in previous studies: 1% in the Free State Province using the RLB hybridisation assay (Mtshali et al., 2013), and, using a nested PCR targeting the rhoptry-associated protein 1 (*rap-1*) gene, 35.5% in the peri-urban localities in Gauteng Province (Mtshali et al., 2014) and 35.1% in all nine provinces of South Africa (Mtshali and Mtshali, 2013). In Mozambique, the prevalence of *B. bovis* was found to be 53% in Maputo Province using a semi-nested hot-start PCR (Martins et al., 2010), much higher than that observed in this study from Zambezia Province (15%). The differences in occurrence of *B. bovis* may be explained by differences in the diagnostic methods used. Alternatively, differences in environment conditions, such as variation in distribution and abundance of the tick vector, *R. microplus*, in geographically separate areas with different climates and topologies, could result in differences in transmission of *B. bovis* (Martins et al., 2010; Mtshali and Mtshali, 2013).

5. Conclusions

Analysis of *B. bovis msa-2b* and 18S rRNA sequences revealed heterogeneity amongst sequences obtained in this study and variation from reference sequences, indicating high diversity of *B. bovis* strains in southern Africa. The *B. bovis msa-2b* phylogenetic clusters identified may not be exhaustive in the study area, given that few samples were sequenced. However, our findings will support further investigations on the genetic diversity of *B. bovis* and evaluation of immunisation of cattle in southern Africa. The 18S rRNA qPCR assay applied in this study reliably detected *B. bovis* in cattle and enabled determination of the infection levels of the parasite in field samples in southern Africa with good precision. The optimised *B. bovis* qPCR assay showed higher detection rates of *B. bovis* than other methods. Therefore, this qPCR, together with a recently reported qPCR for the detection of *B. bigemina* (Stoltsz et al., 2020), are good diagnostic tools for epidemiological investigations and clinical diagnosis, and will contribute to effective control of bovine babesiosis.

CRediT authorship contribution statement

Charles Byaruhanga: Funding acquisition, Investigation, Visualization, Formal analysis, Methodology, Data curation, Conceptualization, Writing – original draft. **S. Marcus Makgabo:** Investigation, Methodology, Writing – review & editing. **Chimvwele N. Choopa:** Resources, Methodology, Writing – review & editing. **Fernando C. Mulandane:** Resources, Writing – review & editing. **Ilse Vorster:** Methodology, Resources, Writing – review & editing. **Milana Troskie:** Resources, Methodology, Writing – review & editing. **Mamohale E. Chaisi:** Methodology, Conceptualization, Writing – review & editing. **Nicola E. Collins:** Funding acquisition, Supervision, Methodology, Conceptualization, Writing – review & editing.

Declaration of Competing Interest

The authors have no conflict of interest.

Data Availability

Data will be made available on request.

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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.2022.102084.

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