Improved detection of *Babesia bigemina* from various geographical areas in Africa using quantitative PCR and reverse line blot hybridisation

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Abstract

Babesia bigemina is one of the aetiological agents of bovine babesiosis, which causes economic losses through mortality, loss of production and control costs. Effective means of detecting and quantifying *B*. *bigemina* in cattle populations is therefore important to inform control approaches. In order to examine the parasite genetic diversity in African countries, *B*. *bigemina*

18S rRNA genes from cattle from South Africa, Uganda and Angola were sequenced. The 25 distinct *B*. *bigemina* 18S rRNA gene sequences obtained in this study showed 99 to 100% identity with previously published sequences of strains from African and other continents. The sequences of the previously published *B*. *bigemina* 18S rRNA gene-specific quantitative PCR (qPCR) primers and probe, developed based on American and Asian strains, were conserved in the African *B*. *bigemina* sequences. The qPCR assay was evaluated using 10-fold and 2-fold serial dilutions of *B*. *bigemina*-infected erythrocytes to determine the efficiency and analytical sensitivity. The qPCR assay had an efficiency of 98.14 ± 1.71%, and the limit of detection was approximately 1.5 infected red blood cells (iRBCs) per microlitre (µl) of blood. The detection rate of *B*. *bigemina* from duplicates of field-collected blood samples from cattle from South Africa, Mozambique and Angola was 37% (30/81), 12% (6/49) and 50% (38/76), respectively. Reverse line blot hybridisation (RLB) results obtained from the same samples in previous studies, using a previously published *B*. *bigemina*-specific probe, detected the parasite DNA in only 1.5% (3/206) of the samples. A new *B*. *bigemina*-specific RLB oligonucleotide probe was designed in the hypervariable V4 region of the 18S rRNA gene. Screening of field blood samples from cattle showed that the new probe was specific, and its frequency of detection of *B*. *bigemina* was three times higher than the previously published probe. The qPCR assay and the newly developed *B*. *bigemina*-specific RLB probe provide good tools for epidemiological studies, which are essential in the control of bovine babesiosis.

Key words**:** 18S rRNA; quantitative PCR; reverse line blot; *Babesia bigemina*; detection; diagnosis

1. Introduction

Babesia bigemina is a tick-borne intra-erythrocytic protozoan parasite of the phylum Apicomplexa, and is a major cause of bovine babesiosis (Bock et al., 2004; De Vos et al., 2004). Bovine babesiosis impacts economically on the cattle industry in tropical and subtropical countries (Bock et al., 2004; De Vos et al., 2004). The costs of the disease are connected with mortalities, decreased milk and meat production, loss of draught power, abortions, control measures, as well as losses of potential production and cattle trade restrictions (Kivaria, 2006; Suarez and Noh, 2011). *Babesia bigemina* is transmitted mainly by the ticks *Rhipicephalus microplus*, *Rhipicephalus annulatus*, *Rhipicephalus decoloratus* and perhaps *Rhipicephalus geigyi*, making it the most widespread bovine *Babesia* species in Africa (Walker et al., 2013).

Unlike *Babesia bovis*, which is characterised by low peripheral blood parasitaemia (less than 1%), *B*. *bigemina* parasitaemias often exceed 10% in acutely-infected animals (Bock et al., 2004). Cattle that recover from the primary acute infection, either naturally or following chemotherapy, remain persistently infected with low parasitaemias, with such animals serving as reservoirs of infection for transmission (Zintl et al., 2005). Although *B*. *bovis* is more virulent than *B*. *bigemina*, killing more than half of susceptible cattle that are infected (Zintl et al., 2005), the distribution of *B*. *bigemina* in southern Africa is wider than that of *B*. *bovis*. In South Africa, for example, *B*. *bigemina* is found across almost the entire country, with the exception of the low rainfall areas of the Northern Cape and Great Karoo. *Babesia bovis* is found only in Limpopo, KwaZulu-Natal, Mpumalanga, Gauteng and around the coast of the Eastern Cape and

the Western Cape (Du Preez and Malan, 2015). The distribution of the parasites corresponds to the presence of their tick vectors; while *B*. *bigemina* is transmitted by both *R*. *microplus* and *R*. *decoloratus* in southern Africa, *B*. *bovis* is transmitted only by *R*. *microplus* (De Vos et al., 2004; Walker et al., 2013). However, *R*. *microplus* is expanding into new areas and displacing *R*. *decoloratus*, therefore epidemics of babesiosis attributed to *B*. *bovis* occur in the region, because local cattle in these areas are naïve to infection (Nyangiwe et al., 2017; Penzhorn, 2018).

Anti-*B*. *bigemina* antibodies have been detected in cattle from various parts of southern Africa: 45.9% in Eastern Cape Province of South Africa (Marufu et al., 2010) and 76.0% in Maputo, Gaza and Inhambane Provinces of Mozambique (Tembue et al., 2011). The prevalences of *B*. *bigemina* using nucleic acid-based techniques have also been documented: 35% in eastern and northeastern areas of Zimbabwe using polymerase chain reaction (PCR) (Smeenk et al., 2000), 76.1% in Gauteng Province in South Africa using nested-PCR (Mtshali et al., 2014), 64.7% in all nine provinces of South Africa using a nested-PCR (Mtshali and Mtshali, 2013), and 61.0% in Maputo Province in Mozambique using a semi-nested hot-start PCR (Martins et al., 2010). Most of the epidemiological studies have shown that the prevalence of *B*. *bigemina* is higher than that of *B*. *bovis*, in areas of southern Africa where both infections occur (Martins et al., 2010; Mtshali and Mtshali, 2013; Mtshali et al., 2014; Sili, 2018), although the economic impact of *B*. *bovis* infection was found to be up to 20-fold higher than that of *B*. *bigemina* in KwaZulu-Natal Province in South Africa (Edwardes, 2019). These reports indicate that bovine babesiosis occurs in many parts of southern Africa, and sensitive and specific diagnostic methods must therefore

be used to detect carrier animals, monitor the prevalence, and understand the epidemiology of the disease, if effective control strategies are to be implemented. In South Africa, the *B*. *bigemina* live blood vaccine is used in the control of babesiosis. Accurate diagnosis is essential to estimate the proportion of naturally exposed cattle, which is an important indicator of the existence of endemic stability, and thus the need to immunise or not (Jonsson et al., 2012).

Advances in nucleic acid-based diagnostic techniques have resulted in the improved detection, identification and genetic characterisation of many haemoparasites using conventional PCR (Oliveira-Sequeira et al., 2005), nested-PCR (AbouLaila et al., 2010), loop-mediated isothermal amplification (Yang et al., 2016), quantitative real-time PCR (qPCR) (Kim et al., 2007) and reverse line blot (RLB) hybridisation (Gubbels et al., 1999; Nijhof et al., 2003). Nucleic acid-based methods have several advantages over serological assays insofar as diagnosis is possible in animals as young as one month of age, and the data obtained by PCR assays reflect the current presence of parasites in circulation (CFSPH, 2008). Furthermore, qPCR assays offer the advantages of reduced contamination risks, shorter time for analysis, robustness, precise quantification of parasite DNA and high sensitivity (Buling et al., 2007; Criado-Fornelio et al., 2009). Due to the higher sensitivity, low or intermittent parasitaemias that may escape detection by other methods may be detected by qPCR (Néo et al., 2016). A TaqMan qPCR has been described for the detection and quantification of *B*. *bigemina* and was designed based on the 18S rRNA genes of American and Asian strains (Kim et al., 2007). It is not known, however, whether this *B*. *bigemina* 18S rRNA gene-specific qPCR assay would effectively detect *B*. *bigemina* from cattle in Africa, given that sequence variation is known to occur amongst *B*.

bigemina parasites (Byaruhanga et al., 2016; Martins et al., 2010). There is therefore a need to determine the effectiveness of the assay for use in research and monitoring of *B*. *bigemina* infections amongst cattle populations in Africa.

The RLB hybridisation assay has been used in Africa and elsewhere in the world to detect mixed haemoparasite infections, including *B*. *bigemina*, in ticks and blood of domestic and wild ruminants. The assay makes use of PCR amplification of haemoparasite rRNA genes, which are screened with group- and species-specific probes for the simultaneous detection and identification of mixed infections of haemoparasites (Bekker et al., 2002; Gubbels et al., 1999; Nijhof et al., 2003, 2005). However, unlike the sequence of the *B*. *bovis* RLB probe (Gubbels et al., 1999), which is conserved in worldwide *B*. *bovis* 18S rRNA sequences, the existing *B*. *bigemina*-specific probe does not detect all genotypes, as observed in blood samples of cattle in Mozambique (Martins et al., 2010) and Uganda (Byaruhanga et al., 2016), where unexpectedly low prevalence of infection was detected in these *B*. *bigemina*-endemic areas using the RLB. This is probably because the RLB probe was designed using only three *B*. *bigemina* 18S rRNA sequences from Mexico (Gubbels et al., 1999), and therefore did not take into account possible sequence variation in other countries.

The objectives of this study, therefore, were three-fold: firstly, to investigate 18S rRNA gene sequence variation amongst *B*. *bigemina* from cattle field samples from African countries, secondly, to assess the *B*. *bigemina*-specific qPCR for detecting *B*. *bigemina* and estimating the

parasite infection levels in cattle from southern Africa, and, thirdly, to design an alternative RLB oligonucleotide probe for improved detection of *B*. *bigemina* in cattle from endemic areas.

2. Materials and Methods

2.1 Ethical statement

The study was approved by the Animal Ethics Committee of the University of Pretoria, South Africa (Project no. V060-17). Blood collections were approved by the Department of Agriculture, Forestry and Fisheries (Pretoria, South Africa) under Section 20 of the Animal Diseases Act of 1984, reference nos. 12/11/1/1/6 and 12/11/1/1/9.

2.2 DNA samples from cattle

DNA samples (n=206) were obtained from previous studies conducted at the Department of Veterinary Tropical Diseases (DVTD), University of Pretoria, from 2015 through 2017. The DNA had been extracted from blood collected from cattle from the Mnisi Community, Mpumalanga Province, South Africa (n=81) (Choopa, 2015), from Namitangurine and Botao villages in the Zambezia Province, Mozambique (n=49) (Nyoni-Phili, 2017), and from Tchicala -Tcholoanga, Huambo Province, Angola (n=76) (Sili, 2018), using a QIAamp® DNA Mini Kit (QIAGEN, Hilden, Germany), and following the manufacturer's instructions.

2.3 Analysis of 18S rRNA sequences from field samples

2.3.1 Amplification of the 18S rRNA gene from samples that tested positive for Babesia on RLB

Thirty-seven samples that had shown positive signals with *Babesia* genus-specific probes (*Babesia* catch-all 1 and/or *Babesia* catch-all 2), with or without *B*. *bigemina*-specific signals,

from RLB analysis were selected for the near full-length 18S rRNA gene (~1600 bp) amplification. Amplification was conducted using the oligonucleotide primers EK-1F (Lefranc et al., 2005) and 18S-1498R (López-García et al., 2003) [Table 1]. The reaction mixtures contained 2.5 µl of DNA, 0.2 µM of each primer, 1X Phusion™ Flash High-Fidelity PCR Master Mix (Thermo Scientific™, LTC Tech South Africa [Pty] Ltd, Randburg, South Africa) 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. PCR-grade water was used as a negative control, while a known *B*. *bigemina* positive sample (*B*. *bigemina* live blood vaccine strain from Onderstepoort Biological Products [OBP], Pretoria, South Africa) was included to serve as a positive control. The PCR products were analysed by electrophoresis on a 2.0% TAE agarose gel.

2.3.2 Cloning and sequencing of PCR products

The 18S rRNA amplicons from all the 37 samples (as in section 2.3.1 above) were purified using the QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany), prior to 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). The clones were screened by colony PCR using primers pJET1.2F and pJET1.2R as described previously (Byaruhanga et al., 2018). Recombinant clones were sequenced at Inqaba Biotechnical Industries [Pty] Ltd (Pretoria, South Africa) with primers pJET1.2F and pJET1.2R and internal sequencing primers RLBF2, RLBR2 and BT18S_3F (Nijhof et al., 2003; Oosthuizen et al., 2008). The 18S rRNA sequences obtained in this study were aligned with other *Babesia* spp. 18SrRNA sequences

Table 1. Oligonucleotide primers and probes used for the detection of *Babesia bigemina* in this study

Assay	Target gene	Oligonucleotide name	Sequence (5'-3')	Amplicon size (bp)	Reference
Reverse line blot (RLB)					
Amplification primers	18S rRNA	RLB-F2	GAC ACA GGG AGG TAG TGA CAA G	520	Nijhof et al. (2003)
		RLB-R2	CTA AGA ATT TCA CCT CTG ACA GT		Nijhof et al. (2003)
	16S rRNA	Ehr-F	GGA ATT CAG AGT TGG ATC MTG GYT CAG	492	Schouls et al. (1999)
		Ehr-R	CGG GAT CCC GAG TTT GCC GGG ACT TYT TCT		Bekker et al. (2002)
Probes[a]		*Babesia* catch-all 1	ATT AGA GTG TTT CAA GCA GAC	-	Nijhof (unpublished)[b]
		Babesia catch-all 2	ACT AGA GTG TTT CAA ACA GGC	-	Nijhof (unpublished)[b]
		B. bigemina probe 1	CGT TTT TTC CCT TTT GTT GG (Old)	-	Gubbels et al. (1999)
		B. bigemina probe 2	GTA GTT GTA TTT CAG CCT CG **(New)[c]**		**This study**
Conventional PCR					
Babesia bigemina	18S rRNA	EK-1F	CTG GTT GAT CCT GCC AG	1,600	Lefranc et al. (2005)
		18S-1498R	CAC CTA CGG AAA CCT TGT TA		López-García et al. (2003)
Quantitative PCR assay					
Babesia bigemina	18S rRNA	BiF	AA TAA CAA TAC AGG GCT TTC GTC T	174	Kim et al. (2007)
		BiR	AAC GCG AGG CTG AAA TAC AAC T		
		BiP probe	VIC - TTG GAA TGA TGG TGA TGT ACA ACC TCA - TAMRA		

[a] Only probes relevant to the detection of *B. bigemina* are shown. The other probes used were as reported in Byaruhanga et al. (2016) and a *Babesia lengau* probe [5'-CTC CTG ATA GCA TTC- 3'] (Bosman et al., 2010)

[b] In Oosthuizen et al. (2009)

[c] A new *B. bigemina*-specific RLB probe designed in this study for world-wide detection of *B. bigemina*

from GenBank using Multiple Alignment with the Fast Fourier Transform (MAFFT) version 7 (Katoh and Standley, 2013). Alignments were examined to determine sequence variation and to examine qPCR primer and probe positions for any mismatches with the *B*. *bigemina* sequences, which could prevent annealing, amplification and detection of species-specific DNA.

2.3.3 Nucleotide sequence accession numbers

The 25 distinct *B*. *bigemina* 18S rRNA sequences obtained in this study have been deposited in GenBank under accession numbers ranging from **MH257699** to **MH257723**.

2.4 Babesia bigemina-specific qPCR assay

2.4.1 Initial qPCR optimisation

The previously reported primer pair BiF-BiR and TaqMan fluorescently-labelled probe BiP (Kim et al., 2007) [Table 1] were used to amplify and detect a 174-bp fragment of the 18S rRNA gene of *B*. *bigemin*a in the qPCR assay. We examined and compared PCR conditions – Master Mix concentration (0.8, 1 and 1.2X), annealing temperature (52, 54, 55, 57 and 60°C), primer concentration (0.4 and 0.5 µM) and probe concentration (0.2, 0.25 and 0.3 µM) – in order to obtain optimal amplification and quantification conditions in our laboratory. Optimisation was performed using DNA extracted from *B*. *bigemina*-infected blood (five samples) and *B*. *bigemina*-infected *R*. *microplus* (two tick samples), confirmed to be *B*. *bigemina*-positive by 18S rRNA gene amplification and sequencing. Each sample was analysed in triplicate. The optimal conditions chosen were those that exhibited the lowest quantification cycles (C_q), least

standard deviation (SD) between C_qs, and with robust fluorescence curves which were parallel to each other in the replicates.

2.4.2 Optimal qPCR conditions

Each PCR reaction contained 0.8X TaqMan® Universal PCR Master Mix (Applied Biosystems, Life Technologies, Johannesburg, South Africa), 2 µl of template (extracted genomic DNA), 0.5 µM of each oligonucleotide primer and 0.25 µM of the VIC and TAMRA-labelled probe 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 thermal cycling at 95°C for 20 s and 57°C for 1 min. Amplification analyses were performed using StepOne™ Software version 2.3 for StepOne™ and StepOnePlus™ Real-Time PCR Systems (Applied Biosystems, Life Technologies, Johannesburg, South Africa). The negative and positive controls for the qPCR assays were as in section 2.3.1 above.

2.4.3 Standard curves and efficiency

To determine the linear range and efficiency of amplification of the qPCR assay, five 10-fold serial dilutions of *B*. *bigemina*-infected bovine erythrocytes (from live blood vaccine strain) were prepared in non-infected bovine blood (ID no. 9193), in order to obtain parasitaemias of 2.67×10^6 to 2.67×10^2 infected red blood cells (iRBC)/ml of blood. All dilutions of the blood vaccine were prepared in triplicate. Genomic DNA was extracted using the QIAamp® DNA Mini

Kit (QIAGEN, Hilden, Germany) from 200 µl of each diluted sample, and the DNA was eluted in AE buffer.

Each dilution was analysed in three replicates in each of six qPCR assay runs to estimate the efficiency of each reaction. Calibration curves were generated by plotting the average C_q values of the dilutions from each qPCR test run against the logarithm of the parasitaemia (log [iRBCs/qPCR reaction]) (2.67×10^4 to 2.67×10^0) for the serial dilutions of blood vaccine. Each qPCR run contained a negative control (PCR-grade water) and a positive control (as in section 2.3.1). The PCR efficiency [E] (Pfaffl, 2001; Vandesompele et al., 2002), expressed as a percentage, was determined by the following formula:

$$\%E = (10^{(-1/Slope)}-1) \times 100$$

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

2.4.4 Limit of detection (analytical sensitivity)

The limit of detection (LOD) of the qPCR assay was determined by preparing a 2-fold dilution series spanning the non-linear range of the standard curve (from section 2.4.3 above), using the *B*. *bigemina* live blood vaccine (2.67×10^2 iRBC/ml) as the starting dilution. The diluent was uninfected bovine blood, confirmed to be negative by RLB hybridisation assay and qPCR. Each dilution series was prepared in quintuplicate and consisted of six separate dilutions, tested in five independent runs (total 25 replicates per dilution). The LOD, determined using the

Statistical Package for the Social Sciences (SPSS) version 25.0 (IBM SPSS, 2017), was defined at input concentration giving a 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.

2.4.5 Estimation of parasitaemia in field blood samples

Field samples were included in duplicate in each of the six qPCR assay runs in section 2.4.3 above, to estimate the parasitaemia (iRBC/µl of blood) from *B*. *bigemina*, using the regression equations from the calibration curves. The total number of field samples from cattle included were 81 from South Africa, 49 from Mozambique and 76 from Angola. Samples that presented a deviation greater than 0.8 of the C_q between duplicates were analysed again.

2.5 New *B*. *bigemina*-specific RLB probe

2.5.1 Probe design and optimal concentration

Because the existing *B*. *bigemina*-specific RLB oligonucleotide probe does not detect all *B*. *bigemina* genotypes from around the world, a new species-specific probe (20 nucleotides) [Table 1] was designed in the hypervariable V4 region of the 18S rRNA gene, specifically targeting an area conserved in, but unique to, all *B*. *bigemina* sequences. To ensure that genetic variation was not overlooked in designing the new probe, we aligned the 18S rRNA sequences obtained in this study with previously published *Babesia* spp. 18S rRNA sequences from all continents (Figure 2) using MAFFT version 7 (Katoh and Standley, 2013). The probe design was screened for parameters required for PCR applications, using OligoAnalyzer 3.1 (Integrated DNA

Technologies [IDT], Inc., Caralville, Iowa, United States; https://eu.idtdna.com/calc/analyser), and subjected to BLASTn search to ensure species specificity to the target DNA sequence. The RLB probe was subsequently synthesised (Integrated DNA Technologies [IDT], Inc., Caralville, Iowa, United States) with a 5AmMC6 binder at the 5' end to facilitate covalent linkage to the RLB membrane (Biodyne® C Membrane, 0.45 µM, Pall South Africa [Pty] Limited, Midrand, South Africa). In order to determine the optimal concentration of the probe for detection of the target, a known concentration of *B*. *bigemina* DNA (live blood vaccine strain, OBP, Pretoria, South Africa) was subjected to RLB analysis with the newly designed probe at three concentrations (2.5, 5.0 and 10.0 µM).

2.5.2 Detection of B. bigemina using the new RLB probe

Field samples from cattle from South Africa, and additional samples from Angola, Mozambique and Uganda (total=20), were tested for *B*. *bigemina* using RLB as previously described (Bekker et al., 2002; Nijhof et al., 2003, 2005). *Theileria*, *Babesia*, *Anaplasma* and *Ehrlichia* genus-specific probes, and 38 species-specific probes were linked to one RLB membrane. Of these probes, 36 were as outlined in Byaruhanga et al. (2016), while the other two probes were a *Babesia lengau*-specific probe [5'-CTC CTG ATA GCA TTC- 3'] (Bosman et al., 2010) and the newly designed *B*. *bigemina*-specific probe. The sequences of the primers used to amplify the V4 region of the 18S rRNA gene (*Theileria*/*Babesia*) and V1 region of the 16S rRNA gene *(Ehrlichia*/*Anaplasma*), and the *Babesia* genus- and species-specific oligonucleotide probes used for the detection of *B*. *bigemina* in the RLB assay are shown in Table 1. DNA from *Anaplasma*

centrale and *B*. *bovis* blood vaccines (OBP, Pretoria, South Africa) were used as positive controls for the 16S rRNA and 18S rRNA PCRs, respectively.

2.5.3 Specificity of the newly designed B. bigemina RLB probe

The newly designed RLB probe was tested for any possible cross-reactions with DNA extracted from other tick-borne parasites, namely *Theileria parva*, *Babesia microti*, *B*. *bovis*, *Babesia occultans*, *Babesia rossi*, *Babesia vogeli*, *Babesia caballi*, *Theileria equi*, *Theileria taurotragi*, *Theileria mutans* and *Theileria velifera*. Specificity was also assessed using negative control DNA from blood of donor cattle (n=11, Onderstepoort Veterinary Research Institute (OVRI), Pretoria, South Africa) raised in tick-free herds, and not vaccinated against or infected with tick-borne infections, and confirmed to be negative by RLB hybridisation assay and qPCR.

3. Results

3.1 *Babesia bigemina* 18S rRNA sequences

Babesia 18S rRNA genes were amplified from 37 field samples from cattle previously shown to be positive for *Babesia* by RLB analysis, with or without *B*. *bigemina* species-specific signals (Choopa, 2015; Nyoni-Phili, 2017; Sili, 2018). Forty-one near full-length *B*. *bigemina* 18S rRNA gene sequences (1,595 bp to 1,671 bp) were obtained from nine of the 37 samples, while 28 samples did not yield any *B*. *bigemina* sequences. Pairwise comparison and BLASTn homology searches revealed that some *B*. *bigemina* 18S rRNA sequences determined herein were identical to each other (16 pairs); thus 25 distinct sequences were identified and deposited in GenBank. These sequences exhibited 99 to 100% identity with previously published sequences

of *B*. *bigemina* strains from cattle in India (GenBank accession no. KF606864), Brazil (FJ426361), Uganda (KU206292), Spain (DQ785311), China (JX495402), and the *B*. *bigemina* chromosome III sequence (LK391709). Inspection of the *B*. *bigemina* 18S rRNA sequences obtained in this study, and those previously published, showed no mismatches in the target sequences of the qPCR forward and reverse primers or the probe designed by Kim et al. (2007).

3.2 *Babesia bigemina*-specific qPCR assay

3.2.1 Optimisation

The qPCR assay showed optimum amplification and quantification of *B*. *bigemina* DNA at an annealing temperature of 57°C, as compared to 55°C reported by Kim et al. (2007). Optimal amplification was realised at concentrations of 0.8X for the Master Mix, 0.5 µM for primers and 0.25 µM for the probe. There was an average C_q difference of 0.52 and a difference in SD of 0.35 between the optimised conditions and the previously published conditions. On evaluation of the *B*. *bigemina* qPCR assay using DNA extracted from *B*. *bigemina*-infected ticks, the ΔR_n [(fluorescence of the reporter dye/fluorescence of a passive reference dye)-baseline], which is an indicator of PCR efficiency, was higher at an annealing temperature of 57°C (ΔR_n = 1.0) than that at 55°C (ΔR_n = 0.05). Furthermore, there was improved sensitivity in detecting *B*. *bigemina* in replicates of DNA samples extracted from ticks at the optimised conditions (average C_q = 34.1 and SD of C_q 0.16 versus average C_q 37.2, SD 0.51 using the previously published conditions).

3.2.2 Standard curves and efficiency

The standard curves obtained from dilutions of infected blood were linear over four logs in the range of 2.67 x 10^4 to 2.67 x 10^1 iRBC/qPCR reaction, enabling quantification of the *B*. *bigemina* parasitaemia in that dilution range. The TaqMan qPCR assay was efficient (average 98.14% ± 1.71) in the amplification of the 174-bp fragment of the *B*. *bigemina* 18S rRNA gene. There was little deviation in efficiency between six replicate qPCR assays, as indicated by mean standard deviations obtained from quantification of *B*. *bigemina* DNA (Table 2). The diagnostic sensitivity and specificity of this qPCR assay were previously evaluated in comparison with a nested-PCR assay based on the *spe1-ava1* fragment (Figueroa et al., 1992), and were both found to be 100% (Kim et al., 2007).

Table 2. Quantitative PCR efficiency determined from six assays performed to estimate *Babesia bigemina* parasitaemia (infected red blood cells)

Assay[a]	Efficiency	Coefficient of determination	Slope	y-intercept
1	99.84	0.9876	-3.3257	40.21
2	95.17	0.9912	-3.4435	40.45
3	99.30	0.9877	-3.3389	40.29
4	97.36	0.9900	-3.3868	40.53
5	98.09	0.9896	-3.3685	40.42
6	99.08	0.9915	-3.3442	40.26
Mean ± SD	98.14 ± 1.71	0.9896 ± 0.0017	-3.3679 ± 0.043	40.36 ± 0.125

[a] Six standard curves were generated from six separate assays. Each curve was obtained by plotting the average quantification cycles (C_q) from three replicates in each qPCR run against the logarithm of the input 10-fold dilution series of equivalent 2.67 x 10^4 to 2.67 x 10^1 *B*. *bigemina* infected red blood cells per qPCR reaction. The 10-fold dilutions were obtained by diluting *B*. *bigemina* live blood vaccine of known parasite concentration in non-infected bovine blood.

3.2.3 Limit of detection or analytical sensitivity of B. bigemina qPCR assay

The LOD, determined by probit analysis, and interpreted at the 95% detection rate amongst the 2-fold replicates was approximately 3 iRBCs/qPCR reaction (Figure 1), corresponding to 1.5 iRBCs/µl of blood or 0.000025% parasitised erythrocytes. This equates to a C_q of 38.97; samples with a higher C_q were considered negative.

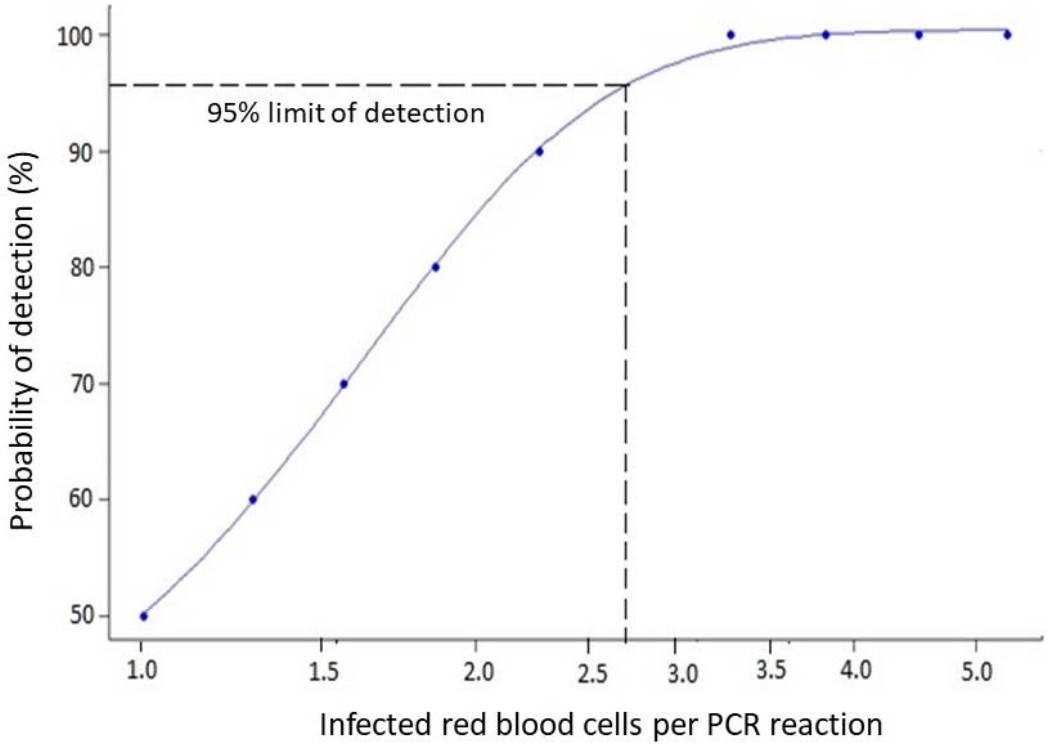

Figure 1. Probit analysis of two-fold dilution series prepared from a 10-fold dilution of *Babesia bigemina* infected erythrocytes, which covered the non-linear range of the qPCR assay, in non-infected bovine blood. The points on the sigmoid curve represent the detection probability derived from 25 replicates (five independent runs with five replicates per run) for each serial dilution. The 95% limit of detection is indicated by the line crossing the sigmoid curve: approximately 3 infected red blood cells per PCR reaction, corresponding to 1.5 iRBCs/µl of blood or 0.000025% parasitised erythrocytes.

3.2.4 Detection of B. bigemina from field samples and quantification of parasitaemia

The assay detected *B*. *bigemina* parasite DNA in 37% (30/81) of samples from South Africa, 12% (6/49) of samples from Mozambique, and 50% (38/76) from Angola. The range of *B*. *bigemina* parasitaemia in the infected cattle was 3 iRBC/qPCR reaction to 58,688 iRBC/qPCR reaction (C_q range of 38.97 to 24.3) or 1.5 iRBC/µl to 29,344 iRBC/µl of blood. This equates to 0.000025% to 0.49% parasitised erythrocytes, assuming 6 x 10^6 RBC/µl of blood in bovine (Cornell University, 2014). There was no amplification in the negative controls.

3.3 Newly designed *B*. *bigemina* RLB probe

3.3.1 Oligonucleotide probe sequence

The sequence of the newly designed *B*. *bigemina*-specific RLB probe is shown in Table 1. Sequence alignment revealed that *B*. *bigemina* 18S rRNA sequences from all over the world are conserved in the target region of the newly designed RLB probe (Figure 2). By contrast, the target sequence of the original probe described by Gubbels et al. (1999) differed in one to three positions from most *B*. *bigemina* sequences (14 out of 22) analysed.

3.3.2 Reverse line blot hybridisation using the new B. bigemina probe

The results from screening of randomly selected field samples from cattle using RLB, qPCR and 18S rRNA amplicon sequencing are shown in Table 3. The new *B*. *bigemina*-specific RLB probe enabled detection of *B*. *bigemina* DNA in 13 out of the 20 screened samples, compared with only four samples detected by the previously published RLB probe. All 13 RLB-positive samples also tested positive using the *B*. *bigemina*-specific qPCR. Furthermore, *B*. *bigemina* 18S rRNA

Figure 2. Nucleotide alignment of the V4 hypervariable region of *Babesia bigemina* 18S rRNA sequences from South Africa and Angola determined in this study with previously published 18S rRNA gene sequences of other *B. bigemina* strains. The positions of the previously published and newly designed *B. bigemina*-specific reverse line blot oligonucleotide probes are shown at the top of the alignment, followed by published sequences of *B*. *bigemina* and other *Babesia* species, with the accession number of each sequence indicated next to the species name. Nucleotide differences are shown in black letters on a white background. The numbers on top correspond to the position of the nucleotides in the 18S rRNA sequence alignment of *Babesia* species. While all *B*. *bigemina* 18S rRNA sequences showed identity to the newly designed probe sequence, there was sequence variation of one to three nucleotides in the target region of the previously published probe.

Table 3. Comparison of results obtained using reverse line blot hybridisation (RLB) assay, real-time PCR (*Babesia bigemina*), 18S rRNA gene sequencing for 20 field blood samples during the assessment of the diagnostic capability of a new *B. bigemina* RLB probe

Sample ID[a]	Origin of sample	Hybridisation with original *B. bigemina* RLB probe	Hybridisation with new *B. bigemina* RLB probe	RLB results (43 probes included[b])[c]	qPCR result (C_q-value)	18S rRNA gene sequencing for *B. bigemina*
B. bigemina vaccine	South Africa	Negative	Positive	T/B, B1, *B. bigemina*	+ (22.23)	+
RE14/013	Uganda	Negative	Negative	E/A, *Anaplasma marginale*, *Anaplasma* sp. Omatjenne, T/B, T, B1, *T. mutans*	+ (37.09)	+
RE14/034	Uganda	Negative	Negative	Negative	-	-
C25	South Africa	Negative	Positive	E/A, T/B, T(f[d]), B1, *T. mutans*, *B. bigemina* (new)	+ (28.66)	+
C31	South Africa	Negative	Positive	E/A, T/B, T, *T. mutans*, B1, *B. bovis*(f), *B. bigemina* (new)	+ (32.27)	+
C36	South Africa	Negative	Positive	T/B, T, B1, *B. bovis* (f), *B. bigemina* (new), *T. velifera*	+ (27.82)	+
C37	South Africa	Negative	Positive	E/A, T/B, T, B1, *B. bigemina* (new), *T. velifera* (f)	+ (26.88)	+
C40	South Africa	Negative	Positive	E/A, T/B, T, B1, *T. mutans* (f), *T. velifera*, *B. bigemina* (new)	+ (25.38)	+
C48	South Africa	Negative	Positive	T/B, T, B1, *T. mutans*, *T. velifera*, *B. bigemina* (new)	+ (29.75)	+
C114	South Africa	Negative	Positive	E/A, T/B, T (f), B1, *B. bovis*, *T. mutans*, *B. bigemina* (new)	+ (32.58)	+
C124	South Africa	Faint positive	Positive	E/A, T/B, T, B1, *T. mutans*, *T. velifera*, *B. bigemina* (new), *B. bigemina* (old) (f)	+ (35.16)	+
C35	South Africa	Negative	Negative	Negative	-	-
67	Angola	Faint positive	Faint positive	E/A, T/B, T, B1, *Theileria* sp. (sable) (f), *T. mutans*, *T. velifera*, *B. bigemina* (new) (f), *B. bigemina* (old) (f)	+ (33.51)	+
85	Angola	Negative	Positive	E/A, T/B, T, B1, *T. mutans*, *T. velifera* (vf), *B. bigemina* (new)	+ (33.24)	+
28	Angola	Faint positive	Faint positive	E/A, *A. centrale*, *A. marginale*, T/B, T, B1, *Theileria* sp. (sable), *T. mutans*, *T. velifera*, *B. bigemina* (new) (f), *B. bigemina* (old) (f)	+ (35.74)	-
73	Angola	Negative	Positive	E/A, T/B, T, B1, *Theileria* sp. (sable), *T. velifera*, *B. bigemina* (new)	+ (31.48)	-
16	Angola	Positive	Positive	E/A, *A. centrale*, T/B, T, B1, *Theileria* sp. (sable), *T. mutans*, *T. velifera*, *B. bigemina* (new), *B. bigemina* (old)	+ (33.54)	+
19	Angola	Negative	Negative	Negative	-	-
5	Mozambique	Negative	Negative	Negative	-	-
11	Mozambique	Negative	Negative	E/A, T/B, T, *T. mutans*, B1, B2, *B. bovis*	-	-

13	Mozambique	Negative	Negative	E/A, T/B, T, *T. mutans*, B1, B2 (f), *B. bovis*	-	-

[a] Samples originated from South Africa (Mpumalanga Province), Uganda (Karamoja Region) and Angola (province of Huambo) and Mozambique (Zambezia Province).

[b] Oligonucleotide probes added to the RLB membrane were: five genus-specific probes (*Theileria*, *Babesia*, *Anaplasma* and *Ehrlichia*) including *Babesia* catch-all 1 and *Babesi*a catch-all 2 (Nijhof, unpublished: in Oosthuizen et al., 2009), 36 species-specific probes as in Byaruhanga et al. (2016), a *Babesia lengau* probe [5'-CTC CTG ATA GCA TTC- 3'] (Bosman et al., 2010) and the newly designed *B. bigemina*-specific probe.

[c] Abbreviations indicate that a hybridisation signal was obtained with the following probes: T/B, *Theileria*/*Babesia* group-specific; E/A, *Erhlichia*/*Anaplasma* group-specific; T, *Theileria* genus-specific; B1, *Babesia* genus-specific 1; *Babesia* genus-specific 2

[d] Indicates a faint hybridisation signal

+, positive; -, negative

sequences, which were closely related (99 to 100% identity) to *B*. *bigemina* sequences published in GenBank, were obtained from 11 of these samples.

3.3.3 Specificity of the newly designed B. bigemina-specific RLB probe

The new *B*. *bigemina* species-specific RLB probe was shown to be specific in its ability to amplify and detect *B*. *bigemina*. No cross-reactions were observed when DNA extracted from other tick-borne parasites were analysed using RLB. Moreover, there was no amplification of DNA from cattle known to be free of tick-borne parasites. Comparison of the sequence of the new *B*. *bigemina* RLB probe with other *Babesia* spp. 18S rRNA sequences in GenBank showed several mismatches to prevent non-specific amplification of other *Babesia* spp. (Figure 2).

4. Discussion

In this study, we describe *B*. *bigemina* 18S rRNA sequence analysis, detection and quantification of *B*. *bigemina* from cattle from southern Africa using a species-specific TaqMan qPCR assay, and detection of the pathogen using a new species-specific RLB oligonucleotide probe.

A qPCR assay has been developed previously for the detection of *B*. *bigemina* (Kim et al., 2007), but was based only on sequences from *B*. *bigemina* strains from America and Asia. Given that sequence variation in *B*. *bigemina* 18S rRNA genes is known to affect the ability of the reverse line blot (RLB) to detect this parasite in African cattle samples (Byaruhanga et al., 2016; Martins et al., 2010), the ability of the qPCR assay to detect all *B*. *bigemina* strains from Africa should be evaluated. For that reason, we sequenced *B*. *bigemina* 18S rRNA genes from field blood

samples of cattle, and compared the new sequences with those available in GenBank, to ensure that the primers and probes specifically amplified and detected *B*. *bigemina* parasite DNA from locally collected field samples. The target sequences of the qPCR primers and probe were conserved in all *B*. *bigemina* strains examined, and should thus enable detection of *B*. *bigemina* infections from a wide geographical range.

Increasing the qPCR primer annealing temperature from 55 to 57°C improved detection of *B*. *bigemina* in both tick and blood samples. This can be explained by the lower specificity that occurs at lower temperatures; at lower temperatures, partial binding of primers to small random locations of similarity can occur in non-specific DNA templates, which may be present in the reaction (such as tick or mammalian host DNA), thus reducing the efficiency in amplification of the target DNA. Non-specific binding is reduced as the annealing temperature is increased. This was particularly noticeable for detection of *B. bigemina* in DNA extracted from ticks, where reduced amplification was found at the lower annealing temperature of 55°C, while the amplification was much improved at 57°C.

The qPCR assay showed reliability for detecting and estimating parasitaemia in field samples. There was generally low parasitaemia in the field samples from cattle, which could indicate carrier status amongst cattle. This persistent infection suggests that there is continuous transmission of *B*. *bigemina* in the cattle population by the tick vectors. The proportion of *B*. *bigemina* positive cattle from South Africa (37%) was higher than that reported by Mtshali et al. (2013) from the Free State Province (3%) using RLB assay, but lower than reported from the

peri-urban localities in Gauteng Province [76.1%] (Mtshali et al., 2014) and from all the nine provinces of South Africa [64.7%] (Mtshali and Mtshali, 2013), using the rhoptry-associated protein-1a (RAP1a) gene nested-PCR. In samples from Mozambique, the frequency of detection of *B*. *bigemina* in cattle from Zambezia Province observed in this study was only 12%, and this was lower than the 61.0% reported from Maputo Province using a semi-nested hot-start PCR (Martins et al., 2010). The variability in *B*. *bigemina* infection rates may be attributed to variation in distribution and abundance of the tick vectors *R*. *microplus* and *R*. *decoloratus* from geographically separate areas with different climates and topologies, which could lead to non-uniform transmission of *B*. *bigemina* (Martins et al., 2010; Mtshali and Mtshali, 2013; Terkawi et al., 2011). For example, in Mozambique, unlike Zambezia Province that shares a border only with Malawi, Maputo Province shares borders with both Swaziland and South Africa; therefore, cattle imports along the borders may increase the likelihood of cross-border spread of *R*. *microplus* ticks and transmission of *B*. *bigemina* amongst cattle.

We developed an alternative *B*. *bigemina*-specific oligonucleotide probe in order to detect genetically distinct *B*. *bigemina* strains in RLB analysis. The previously published oligonucleotide probe for the RLB assay was designed from only three *B*. *bigemina* 18S rRNA sequences from Mexico (Gubbels et al., 1999). As a result, false negative results with regard to the detection of *B*. *bigemina* during RLB analysis have been reported in previous studies (Byaruhanga et al., 2016; Martins et al., 2010), due to nucleotide differences between the RLB probe and *B*. *bigemina* sequences from other countries. Reverse line blot analysis of blood samples from cattle showed that the newly designed *B*. *bigemina*-specific probe improved the detection of *B*.

bigemina, hybridising with 65% (13/20) of the samples tested, in marked contrast to the original probe that detected *B*. *bigemina* in only 20% (4/20) of the samples. This demonstrates the usefulness of the new RLB probe in the detection and identification of *B*. *bigemina*, and its importance in epidemiological studies. Only one sample, RE14/013, which was positive with qPCR, did not hybridise with the new RLB probe, probably due to the low parasitaemia in that sample (as evidenced by the C_q value of 37.1). This can be expected, given that qPCR is generally more sensitive than RLB in detecting pathogen DNA (Chaisi et al., 2017). The newly designed RLB probe was highly specific; no cross-reactions with bovine DNA or any other tick-borne parasites were observed.

The *B*. *bigemina* qPCR assay and the newly developed RLB probe provide powerful diagnostic tools for the detection of carrier and sub-clinical infections, which is important in epidemiological studies to provide useful information for making effective control decisions that can curtail cattle mortalities emanating from bovine babesiosis. The assays could be useful in estimating the level of risk posed, gauging the efficacy of parasite control programmes implemented, and monitoring endemic stability in cattle, the breakdown of which leads to the occurrence of serious outbreaks of babesiosis in endemic areas (Norval et al., 1983; Smith et al., 2000).

Although *B*. *bigemina* is generally more prevalent than *B*. *bovis* amongst cattle in southern Africa (Martins et al., 2010; Mtshali and Mtshali, 2013; Mtshali et al., 2014; Sili, 2018), the latter causes a more severe disease, and given the expansion of *R*. *microplus* to new areas (Nyangiwe

et al., 2017), *B*. *bovis* infections are likely to cause a greater economic impact (Edwardes, 2019). Therefore, there is need also to improve detection methods for *B*. *bovis*. The sequence of the *B*. *bovis*-specific RLB probe is conserved in *B*. *bovis* 18S rRNA sequences published thus far from various regions of the world, and the test can therefore detect all *B*. *bovis* genotypes known to date. However, it is not yet known whether the qPCR assay can detect all *B*. *bovis* genotypes from Africa. Therefore, there is a need also to validate the *B*. *bovis*-specific qPCR assay in African countries, as this will be useful to scrutinise outbreaks of babesiosis in the region.

5. Conclusions

We analysed *B*. *bigemin*a 18S rRNA gene sequences, assessed a qPCR assay for the detection and quantification of *B*. *bigemina*, and developed a new species-specific RLB probe for improved detection of the pathogen. The qPCR assay and the newly designed RLB probe are specific for *B*. *bigemina*, and are broadly applicable to strains of the parasite from diverse geographical regions. The assays, therefore, have application for diagnosis of *B*. *bigemina* infections and monitoring of clinical cases and carrier animals, as well as detection in ticks, even in mixed infections. In addition, this study has expanded our current knowledge concerning the occurrence of cattle infection with *B*. *bigemina* in southern Africa.

6. Conflict of interest

The authors have no conflict of interest.

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