Development and analytical validation of a multiplex, real-time PCR assay for *Babesia rossi* and *Babesia vogeli*

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Abstract

Canine babesiosis is caused by tick-transmitted intraerythrocytic protozoan parasites occurring worldwide. In southern Africa, babesiosis is caused by *Babesia rossi* and *B. vogeli* and is one of the most common and important infectious diseases affecting dogs. There is no reliable, rapid and sensitive method for the detection of these parasites, especially when parasitaemia is low. The aim of this study was to develop a sensitive and specific multiplex TaqMan® MGB PCR assay for the diagnosis of canine babesiosis infections occurring in southern Africa, and to discriminate between *B. rossi* and *B. vogeli*. The fitness of purpose of the assay was to confirm diagnosis of suspect or clinical cases, and estimate prevalence of infection for research purposes.

A total of 648 published sequences were used to design the assay. A set of group-specific canine *Babesia* spp. primers were designed to amplify a 117 nucleotide region of the 18S rRNA gene of all canine *Babesia* spp. Species-specific TaqMan® MGB probes were developed for *B. rossi, B. vogeli, B. canis* and *B. gibsoni*, but analytical validation was only performed for *B. rossi* and *B. vogeli* as a multiplex assay.

The assay had a broad dynamic range and amplified *B. rossi* and *B. vogeli* efficiently (98.6% and 94.7% respectively). The assay was sensitive, with a 95% LOD of $10^{-2.67}$% parasitized erythrocytes (PE) for *B. rossi* and $10^{-2.03}$% PE for *B. vogeli*, and specific, with no cross reaction between *B. rossi* and *B. vogeli* and no detection of other haemoparasites that infect dogs, such as *Ehrlichia canis* and *Anaplasma platys*. Consistent repeatability within and between PCR runs was shown.

This assay will be able to accurately and rapidly confirm babesiosis in canines and allow for treatment to be administered in the early stages of the disease, speeding up the recovery time in affected dogs.

Keywords

Babesiosis, biliary, protozoa, hemoparasite, canine, TaqMan®

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1. Introduction

Babesiosis, first described in cattle and sheep in 1888 (Babes, 1888), is a tick-transmitted infectious disease occurring worldwide in a variety of vertebrate hosts (Homer et al., 2000). The presence of *Babesia canis* (*Pyrosoma bigemina* var. *canis*) was first described by Piana and Galli-Valerio (1895) in a hunting dog in Italy.

Canine *Babesia* species can be classified according to their morphological appearance under light microscopy into large *Babesia* (5 x 2.5 µm) and small *Babesia* (2 x 1.5 µm) species (Levine, 1988). The large canine *Babesia* spp. have similar morphology but infect different vectors. They were subdivided into three sub-species by Uilenberg et al., (1989): *Babesia canis canis* (Piana and Galli-Valerio, 1895), *Babesia canis rossi* (Nuttall, 1910) and *Babesia canis vogeli* (Reichenow, 1937). Based on molecular differences, the three taxa are now referred to commonly as *B. canis, B. rossi* and *B. vogeli* (Carret et al., 1999; Zahler et al., 1998). In addition, another large, unnamed *Babesia* spp. has been described in an immunocompromised dog (Birkenheuer et al., 2004). The small *Babesia* parasites consist of three morphologically similar, but genotypically distinct parasites: *B. gibsoni* Asia type, found in north and eastern Africa and North America (Patton, 1910), "*Theileria" annae*, a *Babesia* parasite of dogs in Spain (Garcia, 2006) and *B. conradae*, identified in dogs in California (Kjemtrup et al., 2006).

Babesia species are transmitted by tick vectors and are strictly vector specific. The presence of the parasite is dependant of the distribution of the tick vector (Uilenberg, 2006). *Rhipicephalus sanguineus* has a worldwide distribution and transmits *B. vogeli. Dermacentor* ticks transmits *B. canis* in Europe (Dantas-Torres, 2008). In southern Africa, *Haemaphysalis elliptica* transmits highly pathogenic *B. rossi*, the most widespread and economically important tick-borne disease in dogs in the region (Collett, 2000) and *R. sanguineus* transmits the less virulent *B. vogeli* (Matjila et al., 2004).

Based on the severity of the infection and the clinical signs, canine babesiosis can be classified as uncomplicated or complicated (Jacobson and Clark, 1994). Complicated babesiosis can lead to a systemic inflammatory response syndrome or multiple organ

dysfunction syndrome, which may present as acute renal failure, hepatopathy, coagulopathy, hypotension, acute respiratory distress syndrome, haemoconcentration, acid-base balance disturbance, acute pancreatitis, neurological signs and cardiovascular system dysfunction (Conrad et al., 1991; Wozniak et al., 1997; Jacobson and Clark, 1994; Welzl et al., 2001; Leisewitz et al., 2001; Matijatko et al., 2009; Schetters et al., 2009).The diagnosis of *Babesia* spp. infections is made most commonly on the size and morphological appearance of the intra-erythrocytic forms of the parasite in peripheral blood smears (Van Heerden et al., 1983). This is a simple and inexpensive method of detection, but has low sensitivity when parasitaemia is low in atypical and chronic cases (Scurell, 2006). This method is also subjective, labour intensive and time-consuming (Bose et al., 1995). Several molecular techniques, including conventional polymerase chain reaction (PCR) (Ano et al., 2001; Birkenheuer et al., 2003; Földvári et al., 2005; Oyamada et al., 2005; Solano-Gallego et al., 2008), real-time PCR (Costa-Júnior et al., 2012) and reverse line blot hybridization (RLB) assays (Gubbels et al., 1999; Bekker et al., 2002; Nagore et al., 2004) have been developed for diagnosing *Babesia* infections in dogs. These techniques have improved the sensitivity and specificity of diagnosis.

Since the development of the polymerase chain reaction in 1986 (Mullis et al., 1986), the technique has developed in flexibility and efficacy, to simultaneously detect several DNA targets with a combination of primers and probes. Conventional PCR enabled the detection of the qualitative presence or absence of a specific DNA sequence (Loftis and Reeves, 2012) and has been described in several studies to distinguish *Babesia* spp., including distinguishing *B. gibsoni* from *B. canis, B, vogeli* and *B. rossi* (Birkenheuer et al., 2003); discriminating between *B. canis, B. vogeli* and *B. rossi* (Duarte et al., 2008) and differentiating *B. canis* from *B. vogeli* (Annoscia et al., 2017).

More recently, quantitative real-time PCR is used to detect a specific DNA sequence in a sample, as well as to determine the actual copy number of this DNA sequence according to standards, simultaneously (Kaltenboeck and Wang, 2005). This is made possible by using dyes (e.g. SYBR-Green) that fluoresce when bound non-specifically to double-stranded DNA (Higuchi et al., 1993), or using fluorescent-labelled DNA probes, which anneal to the target DNA and improves the specificity of the assay (Heid et al., 1996). The cycle at which this accumulation of fluorescence exceeds the background is known as the C_T, which is inversely proportional to the target amount of nucleic DNA in the sample, making it unnecessary for further post-amplification processes to quantify the amount of DNA amplified.

The RLB assay, a PCR combined with a blotting process is able to detect and identify simultaneously *Theileria, Babesia, Anaplasma* and *Ehrlichia* species in a sample. Although the RLB assay, currently performed routinely in the Department of Veterinary Tropical Diseases laboratories, is suitable as a screening tool it requires at least 40 samples to run cost-effectively, is labour intensive and has a slow turnaround time.

Before 1995, all canine babesia parasites were called *B. canis* until the parasite occurring in southern Africa became known as *B. rossi*. *B. vogeli* was confirmed for the first time in domestic dogs in South Africa (Matjila et al., 2004). It is important to differentiate between the different canine babesia parasites in South Africa because the clinical disease caused by *B. rossi* and *B. vogeli* are different. The feeling is that the disease caused by *B. vogeli* is very mild (it has never been described) and the impression is that it is seldom a mono infection usually occurring together with *Ehrlichia canis*.

There is a need to develop a rapid, sensitive real-time PCR assay for the diagnosis of canine babesiosis infections occurring in southern Africa. For critical cases, this needs to be performed in a short as possible turnaround time, to facilitate effective case management. The fitness of purpose of the assay is to confirm diagnosis of suspect or clinical cases, and estimate prevalence of infection for research purposes.

2. Methods

2.1. Assay design

Canine *Babesia* spp. 18S rRNA gene sequences were downloaded from GenBank® (Benson et al., 2013; NCBI, 2017a) and aligned with the online version of MAFFT v7 (Katoh et al., 2002; Katoh, 2013), using default settings. Sequence editing was performed using BioEdit (Hall, 1999). As the quality of the sequences could not be verified, nucleotide (nt) differences that only appeared in a single sequence were ignored, and only differences that occurred in more than two sequences were considered in the design of the primers and probes. Species-specific TaqMan® minor groove binder (MGB) probes and group specific primers were developed with the aid of Primer Express® software for Real-Time PCR v3.0.1 (Applied Biosystems, Thermo Fisher Scientific, USA).

2.2. DNA extraction and multiplex PCR assay

Nucleic acid was purified from 50 µl of ethylenediaminetetraacetic acid (EDTA)-anticoagulated whole blood using the MagMAX™ Total Nucleic Acid Isolation kit (Thermo Fisher Scientific, USA) and MagMAX™ Express Particle Processor (Thermo Fisher Scientific, USA) according to a custom protocol (Appendix A). Two µl Xeno™ DNA Control (10,000 copies/µl)/reaction was added to the MagMAX™ lysis/binding solution concentrate, to serve as a positive internal control for DNA purification and PCR. The nucleic acid was eluted in 50 µl of elution buffer and stored at -20°C, if not analysed immediately.

VetMAX™-Plus qPCR Master Mix (Thermo Fisher Scientific, USA) was used according to the manufacturer's instructions, together with 1 µl VetMAX™ Xeno™ Internal Positive Control - VIC™ Assay and 2 µl (<1 µg) purified nucleic acid. The composition of the buffer and the concentration of the dNTP mix and AmpliTaq Gold® DNA Polymerase of the VetMAX™-Plus qPCR Master Mix is unknown. The reaction was run on the StepOnePlus Real-Time PCR System (Thermo Fisher Scientific, USA) according to the MGB probe thermal cycling

conditions indicated on the manufacturers' instructions for VetMAX™-Plus qPCR Master Mix. The threshold for all reactions was set at 0.1 ΔRn. A no-template control was included in each assay.

Healthy blood samples were taken from donor animals housed at the Onderstepoort Veterinary Academic Hospital, University of Pretoria (OVAH-UP) that tested negative for the presence of haemoparasites by the RLB assay. Convenience sampling was used to collect infected field samples from pet dogs that presented to OVAH-UP and a small animal clinic in Windhoek, Namibia (Penzhorn et al., 2016) for veterinary care. Dogs were selected based on clinical findings classically suggestive of babesiosis and confirmation of the disease on a thin, stained capillary blood smear. Ethical clearance was approved by the University of Pretoria's Animal Ethics Committee (V034-14, V055-11 and V010-07). Blood samples were collected in EDTA Vacutainer® blood collection tubes (BD, USA) and complete blood counts were performed on selected samples by an Advia 2120 automated haematology analyzer (Siemens, Germany)

Field samples were tested in parallel by the RLB and PCR assays. Assuming 95% sensitivity and specificity of the PCR assay, with an allowable error of 5% and a confidence interval of 95%, at least 73 samples from animals of known infection status were targeted for testing (Jacobson, 1998).

2.3. Plasmid controls

Diagnostic samples submitted to OVAH-UP, that tested positive for *B. rossi* (RE 16/016) and *B. vogeli* (RE 15/253) by RLB assay were used to construct plasmid controls.

A 460 – 540 bp region of the 18S SSU rRNA gene, spanning the conserved V4 region of *Babesia* spp. and *Theileria* spp., was amplified using forward primer RLB-F2 (5'-GACACAGGGAGGTAGTGACAAG-3') and reverse primer RLB-R2 (5'-CTAAGAATTTCACCTCTGACAGT-3') (Gubbels et al., 1999). Approximately 75 ng (2 µl) of DNA template was added to a Phusion Flash High-Fidelity PCR master mix containing Phusion Flash II DNA polymerase, dNTP's and $MgCl_2$ (Thermo Fisher Scientific, USA) in a final volume of 25 µl. Amplification consisted of an initial denaturing at 98°C for 10 s, followed by 40 cycles of 98°C for 1 s, 52°C for 5 s and 72°C for 15 s, with a final extension at 72°C for 1 min. The PCR products were purified using the High Pure PCR Product Purification Kit (Roche, South Africa) and quantified using a Trinean Xpose spectrophotometer (Anatech Instruments, South Africa).

The PCR products were cloned into a pGEM®-T Easy Vector (Promega, USA) and transformed into competent JM109 high efficiency *Escherichia coli* cells (Promega, USA) according to the protocol of the manufacturer and plated by using standard techniques onto (two per sample) imMedia Amp Blue culture plates (Invitrogen, USA) and incubated overnight at 37°C.

At least three white colonies per plate were selected and screened simultaneously for the correct DNA insert (~460 - 540 bp) by means of a colony PCR and placed into a Falcon™ bottle (Thermo Fisher Scientific, USA) in 4 ml of imMedia™ Amp liquid broth (Invitrogen, USA) and incubated overnight at 37°C for growth. Colony PCR was performed using the pJET1.2_F primer (5'-CGA CTC ACT ATA GGG AGA GCG GC-3') and pJET1.2_R primer (5'-AAG AAC ATC GAT TTT CCA TGG CAG-3') supplied by the kit and used according to the manufacturer's instructions. Glycerol stocks of each clone was prepared by adding and gently mixing volume/volume of bacterial growth and 50% glycerol solution and stored at -80°C.

Recombinant plasmids were isolated from the overnight bacterial growths by following the manufacturer's instructions of the High Pure Plasmid Isolation KIT (Roche Diagnostics, Mannheim, Germany). Plasmid DNA was eluted in 20 µl elution buffer and the concentration determined by calculating the average of multiple readings using the Trinean Xpose (Anatech Instruments, South Africa) and Biotek Powerwave™ (Analytical and Diagnostic Products, South Africa) spectrophotometers. The copy number (per µl) was calculated by means of the following formula: 6.022×10^{23} (copy number/mol) × concentration (g/µl) / molar mass (g/mol). The plasmid DNA was stored at -20°C.

2.4. Primer and probe optimisation

The TaqMan® PCR assay was designed for the simultaneous detection of *B. rossi* and *B. vogeli*. The probes were labelled with 6-FAM and NED™ dyes respectively, and the Xeno™ probe labelled with VIC™ dye. The primer concentration in the PCR reaction was optimized by testing different primer concentrations (50 nM, 100 nM, 200 nM, 400 nM and 800 nM in the PCR reaction) with a 120 nM probe concentration in the PCR reaction, and 8.9×10^3 *B. rossi* and 9.3×10^4 *B. vogeli* plasmid copies. The primer concentrations were limited, to ensure amplification of all targets in a multiplex reaction. The amplification curves were viewed and the lowest primer concentration that produced an efficient amplification curve (steep slope viewed on a linear scale) with a low cycle threshold (C_T) was selected subjectively. Probe concentrations of 30 nM, 60 nM, 120 nM, 250 nM, 500 nM in the PCR reaction were tested with the selected primer concentration.

2.5. Analytical validation

The efficiency and linear range of the assay was calculated using a ten-fold dilution series of blood (negative for babesiosis by RT-PCR and obtained from an uninfected donor dog) spiked with plasmid control DNA. The dilution series was tested five times in a single run. PCR efficiencies were determined by the formula: PCR efficiency (%) = $100 \times (10^{-1/\text{slope}} - 1)$.

A two-fold dilution series, consisting of eight separate dilutions of blood spiked with plasmid control DNA was made to cover the non-linear range of the assay at the limit of detection. Each dilution was extracted five times and each extract tested in five independent runs. The results of these analyses were used to calculate the 95% limit of detection (LOD) (input

concentration giving a positive result in 95% of the replicates) by probit analysis (SPSS Statistics v25, IBM Analytics, USA). All calculations were performed using four copies of the 18S rRNA gene per parasite (Dalrymple et al., 1992).

The analytical specificity of both assays were evaluated by testing DNA samples extracted from other protozoal parasites, identified by the reverse line blot hybridization assay or by sequencing. The samples used to determine the specificity of the *B. rossi* and *B. vogeli* assays are listed in Table 1.

Table 1. The specificity of the assay was determined by testing the assay against the haemoparasites listed, RE – diagnostic samples submitted to Department of Veterinary Tropical Diseases, University of Pretoria.

Positive DNA identification	Reference
Anaplasma centrale	Blood vaccine (Onderstepoort Biological Products)
Anaplamsa platys	RE 18/052
Babesia bigemina	Blood vaccine (Onderstepoort Biological Products)
Babesia bovis	Blood vaccine (Onderstepoort Biological Products)
Babesia caballi	Tissue culture obtained from Onderstepoort Veterinary Institute as a diagnostic sample
Babesia microti	Zivkovic et al., 1984 or Gray strain
Babesia occultans	Tissue culture obtained from Onderstepoort Veterinary Institute as a diagnostic sample
Ehrlichia canis	RE 18/029
Ehrlichia ruminantium	Blood vaccine (Onderstepoort Biological Products)
Theileria equi	RLB 12/058
Theileria mutans	RLB 18/008
Theileria parva	Buffalo 102, housed at Onderstepoort Veterinary Institute
Theileria taurotragi	RLB 18/008
Theileria velifera	RLB 18/008

The inter-run, intra-run and total standard deviations (SD) were calculated by the formulas: SD of the means of all runs; mean of the SD of all runs; SD of all replicates, respectively. The total coefficient of variation (CV) was calculated by the formula: total CV = total SD/(mean C_T-value of all replicates).

3. Results

3.1. Assay design

A total of 401 B. *canis* 18S rRNA gene sequences were downloaded from GenBank® (Benson et al., 2013; NCBI, 2017a). Sixteen sequences containing internal transcribed spacer sequences were removed, and GQ39395379 and GQ395382 aligned manually, due to difficulty aligning the sequences using MAFFT v7 (Katoh et al., 2002; Katoh, 2013). Four sequences (DQ174282, DQ174287, DQ174289 and KT246306) were removed, as each sequence was only partially identified as a *B. canis* sequence using BLAST® (Altschul et al., 1990; NCBI, 2017b), the rest of the sequence was not identified. The sequence for KC985241 was in the wrong orientation and the reverse complement sequences used. Three

sequences (KF112072, KF112073 and KT248305) were labelled as *B. canis*, but BLAST® (Altschul et al., 1990; NCBI, 2017b) results indicated that they were *B. gibsoni* sequences.

A species-specific region (between nucleotides 598 – 664, using GenBank accession number L19079 as reference) flanked by conserved regions was identified. Sequences with no data in the region of interest (nt 519 – 738) were deleted, which left 363 sequences. The region of interest was trimmed, de-gapped in BioEdit (Hall, 1999) and re-aligned in MAFFT v7 (Katoh et al., 2002; Katoh, 2013).

A total of 247 *B. gibsoni* 18S rRNA gene sequences were downloaded from GenBank® (Benson et al., 2013; NCBI, 2017a). After the sequences were aligned in MAFFT v7 (Katoh et al., 2002; Katoh, 2013), those that did not align within the target region of the assay, or that were in the wrong orientation (HG328235 – HG328237), were deleted, leaving 213 sequences.

Group-specific canine *Babesia* spp. primers and species specific TaqMan® MGB probes were developed for *B. rossi, B. vogeli, B. canis* and *B. gibsoni* (Table 2). The assay targeted a 117 nt region of the 18S gene. The position of the primers and probes, and variation in the sequences in the target region is shown in Fig. 1.

Table 2. A canine *Babesia* spp. species-specific assay, consisting of genus-specific primers (names ending in F/R) and species-specific TaqMan® MGB probes (names ending in P). Accession number L19079 was used for numbering of the start position of the primers/probes, R = A/G.

Name	Sequence	Start position	Length (bp)	Melt temp. (°C)	GC (%)
BDogF	TGTTGCAGTTAAAARGCTCGTAGTT	572	25	59	38
BDogR	AGTCTGCTTGAAACACTCTAATTTTCTC	671	28	59	36
BRossiP	TGGCTTTTTGCCTTATTA	649	18	68	33
BVogeliP	AGTTTGCCATTCGTTTGG	612	18	69	44
BCanisP	TTATTTCGTTTTCGCTTTTGGGAA	631	24	70	33
BGibsoniP	CCCGACTCGGCTACT	±611	15	70	67

3.2. Plasmid controls

Successful cloning of partial 18S *B. rossi* and *B. vogeli* genes were confirmed by RLB assay and this PCR assay. Spectrophotometric analysis showed a concentration of 4.49×10^9/µl *B. rossi* plasmids (1.12×10^9/µl *B. rossi* parasites) and 4.64×10^9/µl *B. vogeli* plasmids (1.16×10^9/µl *B. vogeli* parasites). The red blood cell (RBC) concentration of the blood sample used to make dilutions of the plasmid was 6.59×10^6 cells/µl; other parameters are shown in Table 3.


580 590 600 610 620 630 640 650
660 670 680 690
L19079_Brossi_(7)
AB303075_Brossi_(11)
JN982353_Brossi_(11)
AB303071_Brossi
KF791208_Brossi
LC053449_Bvogeli_(139)
JX871889_Bvogeli_(17)
JX304670_Bvogeli
JX304669_Bvogeli
AJ009796_Bvogeli_(12)
FJ492058_Bvogeli
KJ513203_Bvogeli
AF548006_Bvogeli_(7)
AB303076_Bvogeli
KP896302_Bvogeli_(3)
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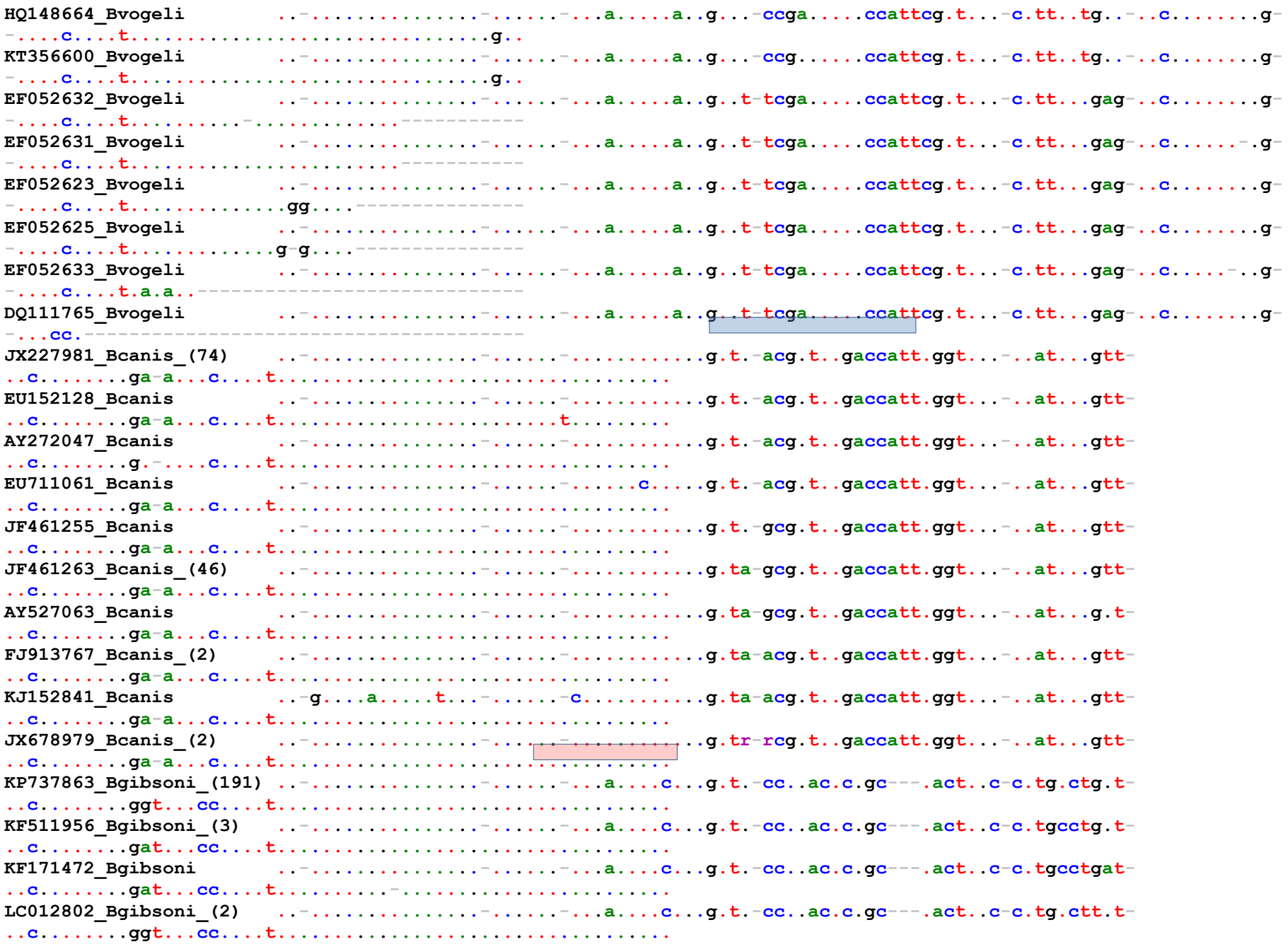
JF500168_Bvogeli


HQ148664_Bvogeli
KT356600_Bvogeli
EF052632_Bvogeli
EF052631_Bvogeli
EF052623_Bvogeli
EF052625_Bvogeli
EF052633_Bvogeli
DQ111765_Bvogeli
JX227981_Bcanis_(74)
EU152128_Bcanis
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AY527063_Bcanis
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KJ152841_Bcanis
JX678979_Bcanis_(2)
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KF511956_Bgibsoni_(3)
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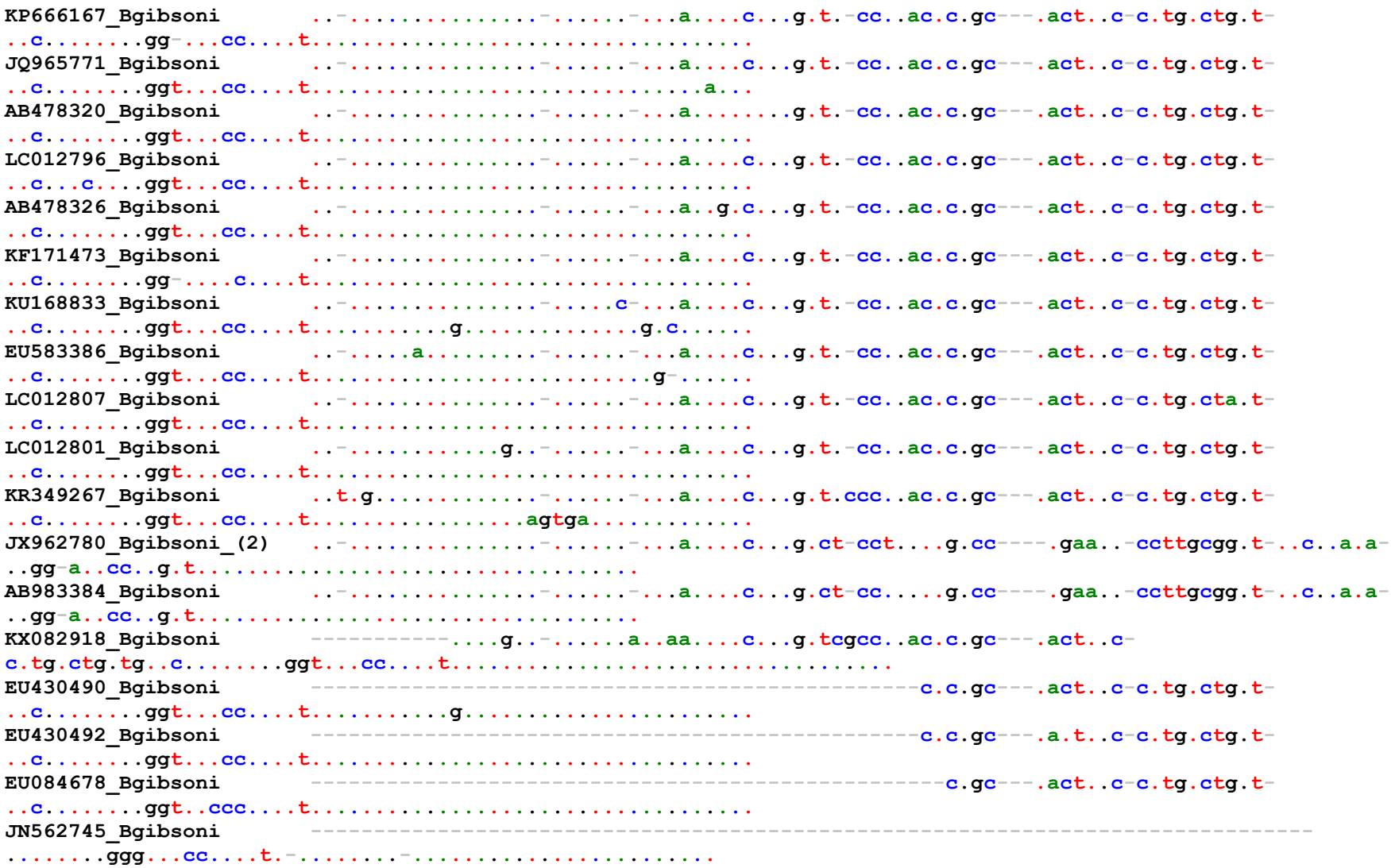


Fig. 1. Position of primers (coloured arrows) and probes (coloured rectangles) for *Babesia rossi* (yellow), *B. vogeli* (purple), *B. canis* (blue) and *B. gibsoni* (red) using L19079 as a reference (dots in the sequence refer to an identical nucleotide to the reference sequence). Titles refer to the Genbank® accession number, the *Babesia* species and the number of sequences (in brackets) in Genbank® (including partial sequences) that shared identical sequences for the region of interest.

Table 3. Selected parameters of a complete blood count of the blood sample used to dilute *B. rossi* and *B. vogeli* plasmid DNA, * - outside of reference range.

	Result	Units	Reference range
White cell count	12.49	$\times 10^3$ cells/µl	6.00 – 15.00
Red cell count	6.59	$\times 10^6$ cells/µl	5.50 – 8.50
Haemoglobin	149	g/L	120 – 180
Haematocrit	0.45	L/L	0.37 – 0.55
Mean corpuscular volume	69.0	fL	60.0 – 77.0
Mean corpuscular haemoglobin	22.7	pg	No reference range
Platelet count	197*	$\times 10^3$ cells/µl	200 - 500

3.3. Primer and probe optimisation

A primer concentration in the PCR reaction of 200 nM was selected as the lowest primer concentration that amplified the targets efficiently. A primer concentration of 200 nM in the PCR reaction was used to test different probe concentration and 250 nM probe concentrations in the PCR reaction for both *B. rossi* and *B. vogeli* probes were selected as the concentration that yielded efficient amplification curves (Fig. 2).

3.4. Analytical validation

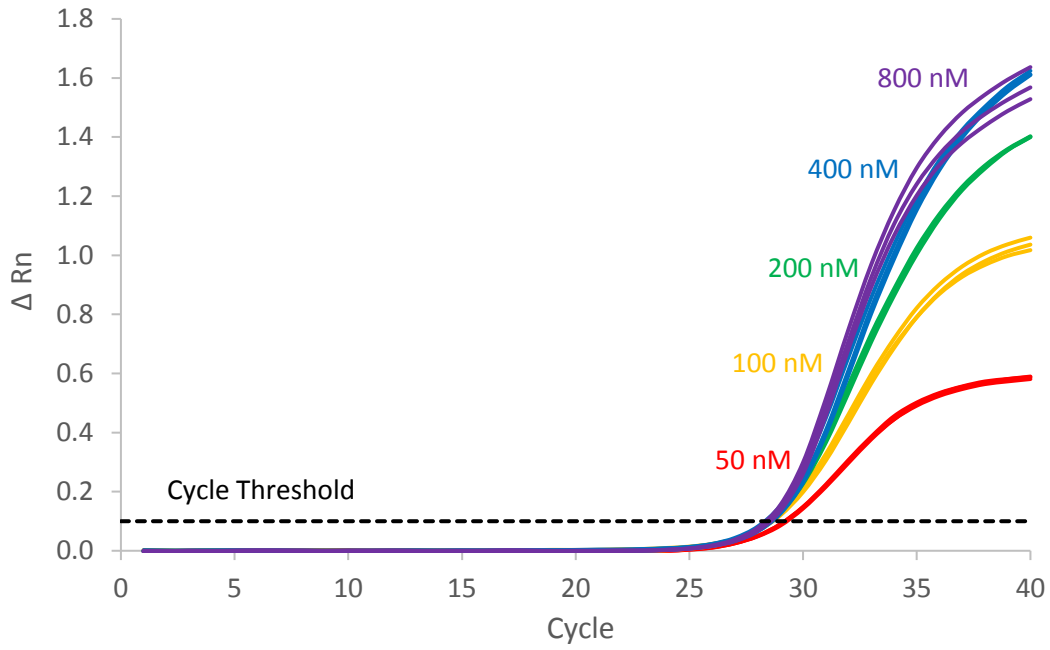
The assays for *B. rossi* and *B. vogeli* were linear between $10^{1.4}$ to $10^{8.4}$ parasites/reaction tested. Linear regression analysis indicated $R^2 > 0.99$ for both assays (Fig. 3). The efficiencies of the assays were calculated at 98.6% and 94.7% for *B. rossi* and *B. vogeli* respectively ([dataset]Troskie et al., 2018).

The assay was sensitive, with a 95% LOD of $10^{-2.67}$% parasitized erythrocytes (PE) (95% confidence interval [CI]: $10^{-2.17}$ – $10^{-2.98}$) for *B. rossi* and $10^{-2.03}$% PE (95% CI: $10^{-1.83}$ – $10^{-2.16}$) for *B. vogeli* (Fig. 4). This was equivalent to 0.21 parasites per 10 000 red blood cells (RBC) (95% CI: 0.1 – 0.67) for *B. rossi* and 0.93 parasites per 10 000 RBC (95% CI: 0.70 – 1.50). This corresponded to a C_T of 36.12 for *B. rossi* and 37.85 for *B. vogeli* ([dataset]Troskie et al., 2018).

Both assays were specific in their ability to detect *B. rossi* and *B. vogeli*. There was no cross reaction between *B. rossi* and *B. vogeli*. There was no amplification from the DNA isolated from other protozoal haemoparasites, such as *Ehrlichia canis* and *Anaplasma platys*.

Both the intra- and inter-run standard deviations (SD) were low, with maxima of 0.70 and 1.02 respectively for *B. rossi* and 0.41 and 1.30 for *B. vogeli* (Table 4). The maximum coefficient of variation (CV) was 3.48, indicating low variation between different replicates and different runs ([dataset]Troskie et al., 2018).

a)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1.6
1.4
1.2
1.0
0.8
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0.4
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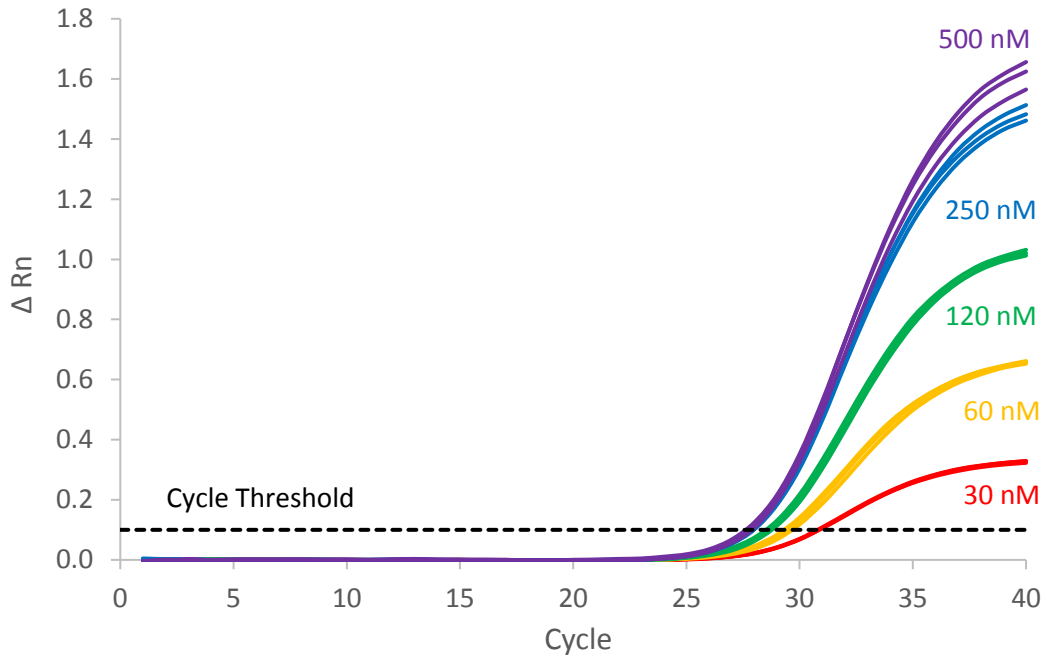
Δ Rn
800 nM
400 nM
200 nM
100 nM
50 nM
Cycle Threshold
0
5
10
15
20
25
30
35
40
Cycle

b)


1.8
1.6
1.4
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0.0
Δ Rn
500 nM
250 nM
120 nM
60 nM
30 nM
Cycle Threshold
0
5
10
15
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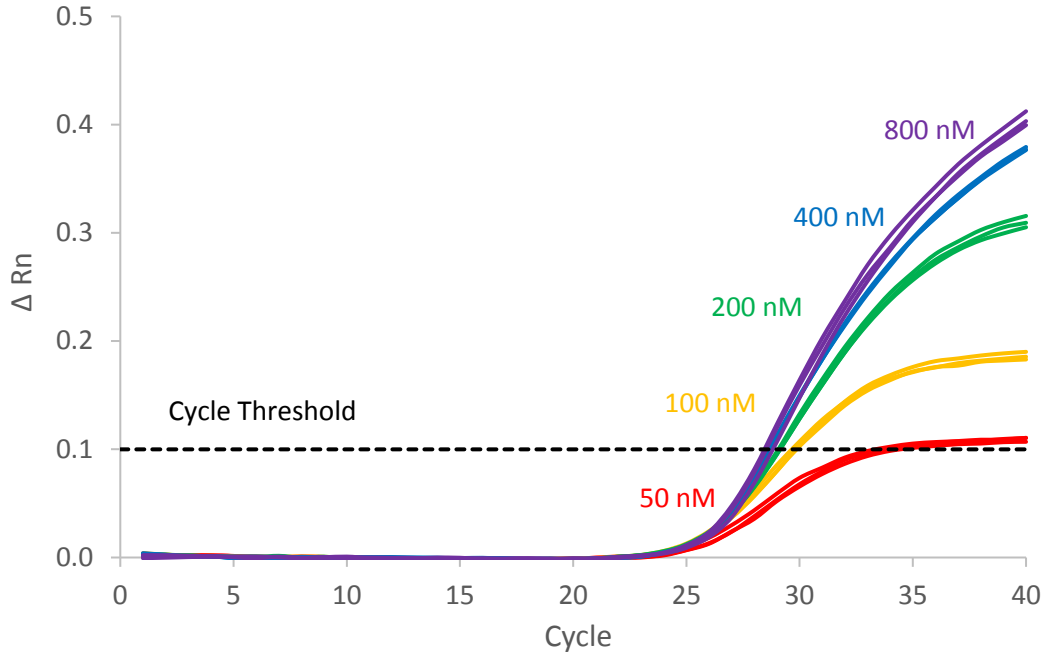
30
35
40
Cycle

c)



d)

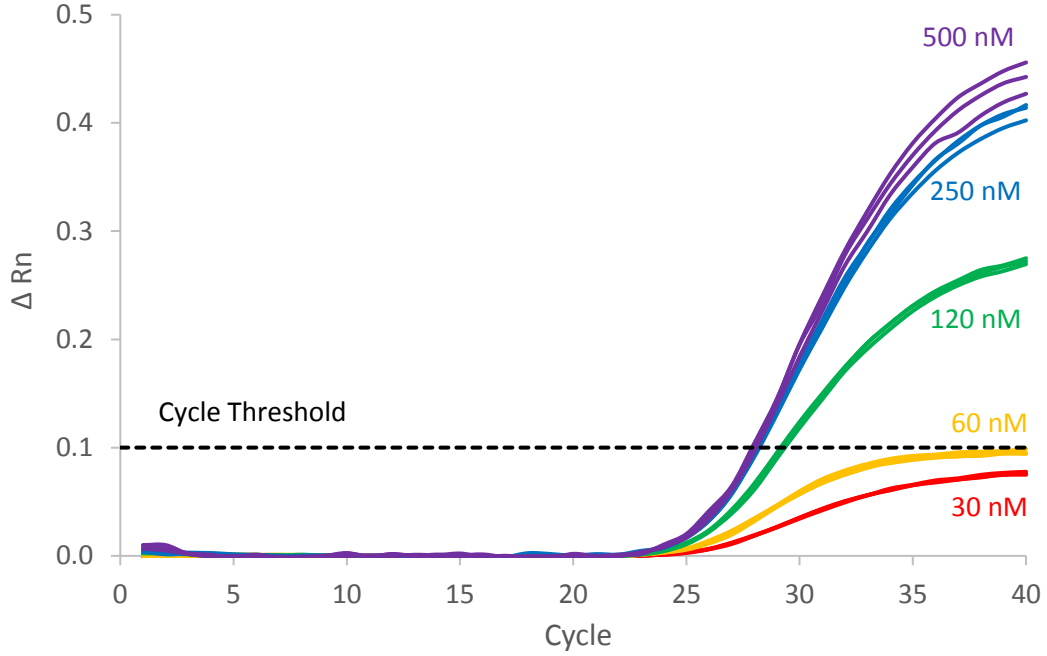
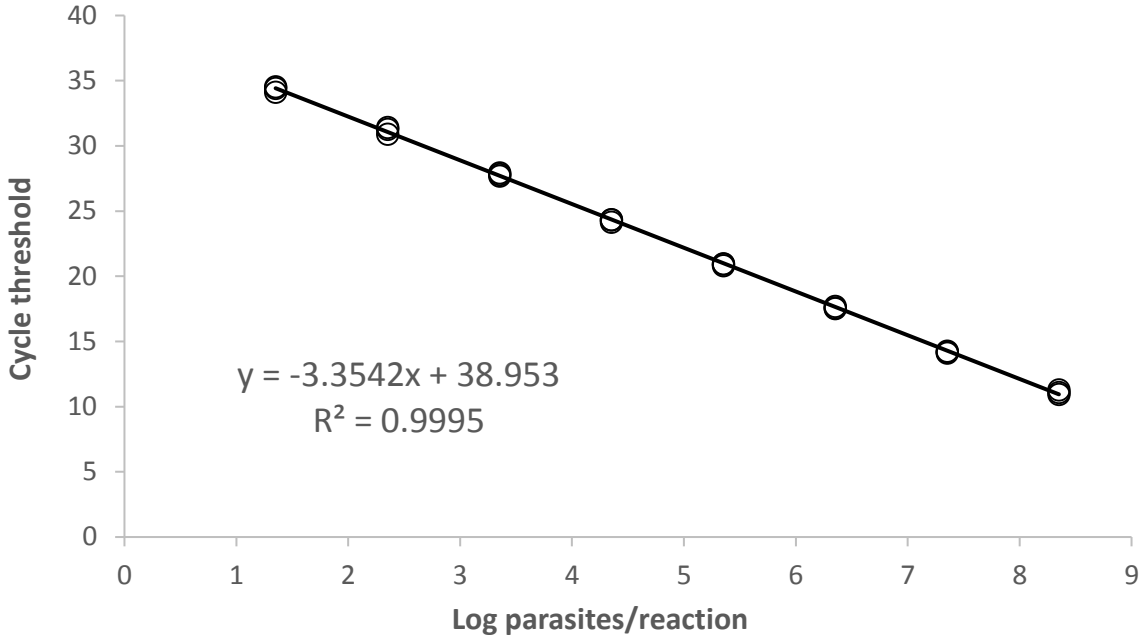


Fig. 2. Optimisation of primers (a and c) and probes (b and d) for *B. rossi* (a and b) and *B. vogeli* (c and d). Primers were optimised using a probe concentration of 120 nM and probes optimised with a primer concentration of 200 nM. A cycle threshold of 0.1 was selected, Δ Rn - reporter signal normalized to the fluorescence signal of ROX™, minus the baseline.

a)



b)

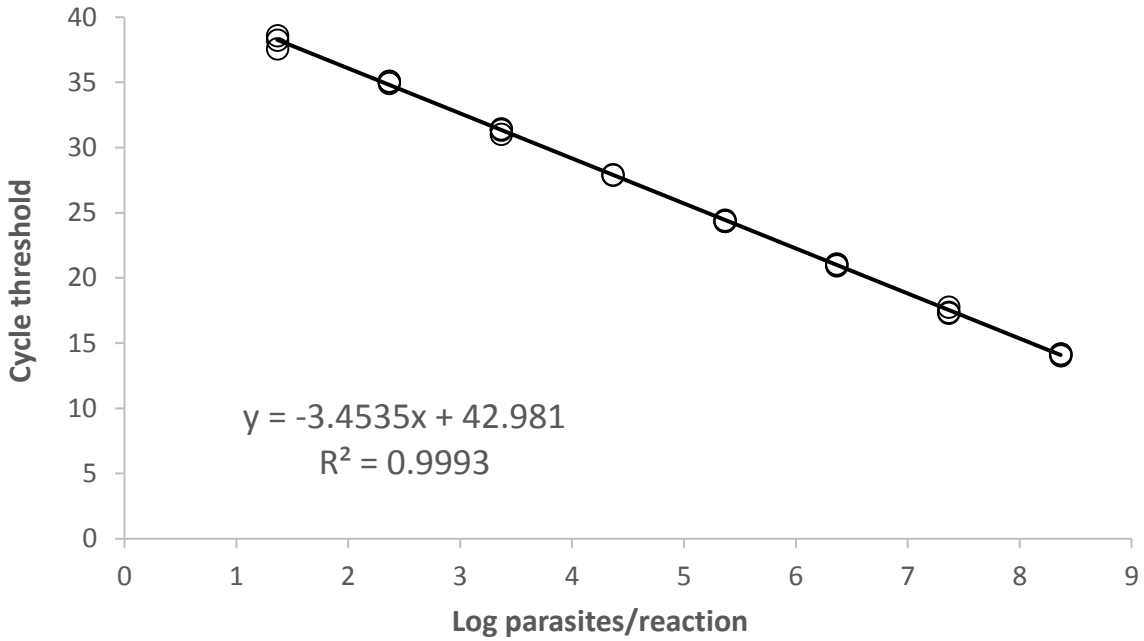


Fig. 3. Ten-fold dilutions of canine blood spiked with plasmid DNA of a) *B. rossi*, b) *B.vogeli* showed linearity over an eight-log range.

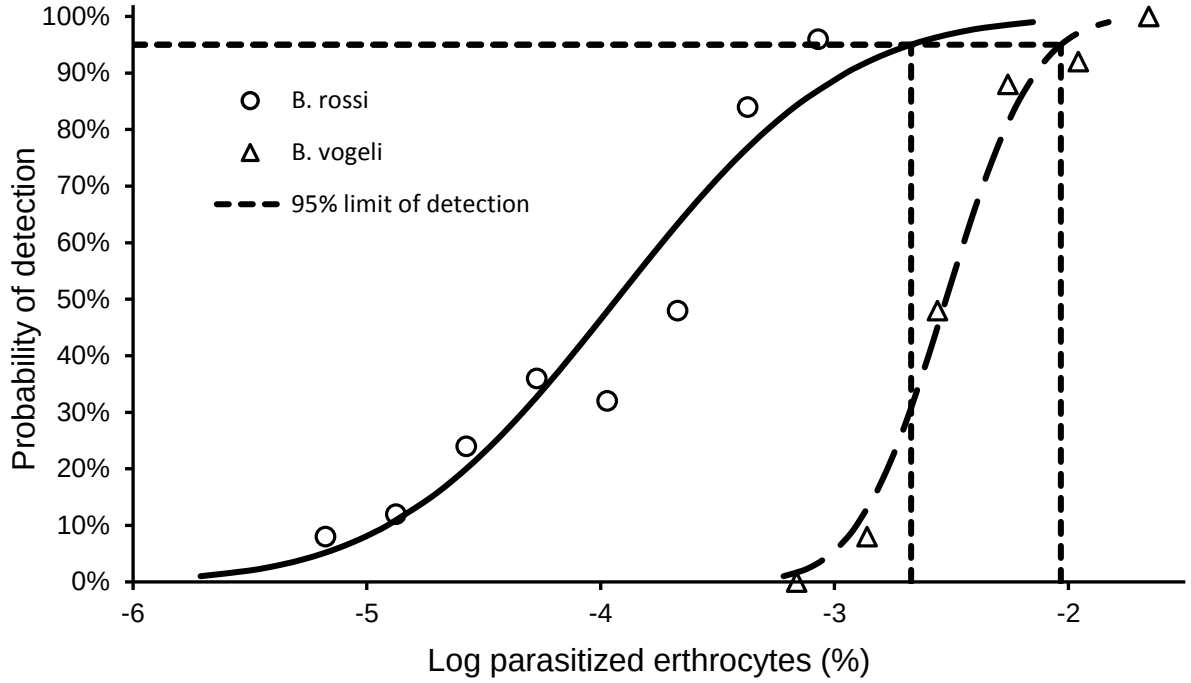


Fig. 4. Two-fold dilution series at the non-linear range of the a) *B. rossi* and b) *B. vogeli* assays were used to calculate the 95% limit of detection.

Table 4. Inter- and intra-run variation for two-fold dilutions of a) *B. rossi* and b) *B. vogeli* infected canine blood, at the non-linear range of the assay. Each sample was analysed five times, on five separate occasions (n = 25), C_T - cycle threshold, Parasitized erythrocytes – PE, SD - standard deviation, CV - coefficient of variation.

a)

PE (%)	Mean (C_T)	Inter-run SD	Intra-run SD	Total SD	Total CV%
1.70×10^{-3}	36.07	0.23	0.80	0.79	2.19
8.51×10^{-4}	36.50	0.28	0.78	0.86	2.37
4.26×10^{-4}	37.11	0.29	0.74	0.69	1.86
2.13×10^{-4}	37.80	0.35	0.72	0.74	1.95
1.06×10^{-4}	37.61	0.64	1.02	1.05	2.78
5.32×10^{-5}	37.83	0.31	0.80	0.53	1.40
2.66×10^{-5}	38.75	0.70	0.78	0.70	1.80
1.33×10^{-5}	38.46	0.23	0.74	0.05	0.13

b)

PE (%)	Mean (C_T)	Inter-run SD	Intra-run SD	Total SD	Total CV%
8.85×10^{-2}	34.91	0.19	1.30	1.21	3.48
4.41×10^{-2}	35.54	0.28	0.27	0.36	1.01
2.20×10^{-2}	36.99	0.25	0.85	0.82	2.23
1.10×10^{-2}	38.40	0.30	0.81	0.79	2.07
5.50×10^{-3}	39.00	0.31	0.56	0.61	1.56
2.75×10^{-3}	39.17	0.41	0.22	0.41	1.06
1.38×10^{-3}	39.51	0.01	-	0.48	1.21

3.5. Diagnostic performance

Blood samples from 121 dogs that presented with clinical signs of babesiosis and were confirmed to be infected with large *Babesia* parasites by visualisation of thin, stained capillary blood smears, and 86 negative control dogs were tested in parallel by the RLB and PCR assays (Appendix B).

For the detection of large canine *Babesia* spp. (*B. rossi* and *B. vogeli*), the RLB assay produced two false-negative and no false-positive results. The diagnostic sensitivity and specificity of the RLB assay was 98.3% (95% CI: 94.2–99.8) and 100% (95% CI: 95.8–100) respectively, with a predictive value of a positive test result (PPV) of 100% and a predictive value of a negative test result (NPV) of 97.7%.

For the detection of large canine *Babesia* spp. (*B. rossi* and *B. vogeli*), the PCR assay produced five false-negative and no false-positive results. The diagnostic sensitivity and specificity of the PCR assay was 95.9% (95% CI: 90.6–98.6) and 100% (95% CI: 95.8–100) respectively, with a PPV of 100% and a NPV of 94.5%.

The sensitivities and specificities of the RLB and PCR assay were compared and no significant differences ($p = 0.25$) were found.

The RLB assay showed that 93.3% of the positive results were diagnosed as a *B. rossi* infection and 6.7% as a *B. vogeli* infection. PCR results showed a mean C_T of 24.94 (standard deviation [sd] = 4.08, n = 109) for *B. rossi* and a mean C_T of 31.49 (sd = 1.95, n = 7) for *B. vogeli*.

The PCR assay was compared to the RLB assay, which was used as the reference assay. For the detection of *B. rossi*, the sensitivity of the PCR assay was 98.2% (95% CI: 93.6–99.8) and the specificity 100% (95% CI: 95.9–100.0), with a PPV of 100% and a NPV of 97.8.8%. For the detection of *B. vogeli*, the sensitivity of the PCR assay was 87.5% (95% CI: 47.4–99.7) and the specificity 100% (95% CI: 95.9–100.0), with a PPV of 100% and a NPV of 95.7%.

Samples identified as positive for *B. rossi* by the RLB assay were used to perform a ROC curve analysis to evaluate the performance of the PCR assay. The area under the curve was 0.99 (95% CI: 0.98–1.00). Using the 95% LOD cut-off C_T of 36.12, the analysis showed a sensitivity of 96.7% and specificity of 98.8%.

4. Discussion

The aim of this study to develop a sensitive species-specific real-time PCR for the diagnosis of canine babesiosis infections occurring in southern Africa was achieved by designing a TaqMan® PCR assay using *Babesia* genus-specific primers and DNA probes specific for B. rossi, B. vogeli, B. *canis* and *B. gibsoni.* Although TaqMan® probes for *B. canis* and *B. gibsoni* were developed, these probes were not tested in this study. TaqMan® MGB probes were selected, as they allow for the design of shorter probes compared to conventional probes, which is useful when conserved regions need to be identified in an area of high variability in

the target region. The probes also have a lower background signal, which results in better precision of the PCR assay.

Babesia canis is transmitted exclusively by *Dermacentor* ticks which do not occur in southern Africa. *Babesia gibsoni* is not endemic to the region, but is a controlled disease in South Africa and all dogs imported from endemic countries (Asia, North America, North and East Africa and Europe), are subjected to pre-import blood tests. The vector for *B. gibsoni* has not yet been determined, although *Haemaphysalis* bispinosa, H. longicornis, H. leachi and *R. sanguineus* sensu lato (Nava et al., 2015) have been implicated in the transmission of the parasite (Wozniak et al., 1997; Kjemtrup et al., 2000). *H. leachi* and *R. sanguineus* sensu lato (Nava et al., 2015) are both endemic to South Africa (Horak, 1995). *Babesia rossi* and *B. vogeli* are the only likely *Babesia* parasites to be found in South Africa (Matjila et al., 2008) and the probes to detect these parasites were tested and validated in the laboratory.

A total of 648 *Babesia* spp. sequences were used to develop the assay, which we believe included most of the canine *Babesia* spp. 18S sequences available publically in databases. The 18S rRNA gene is known to be highly conserved within each *Babesia* species (Birnstiel et al., 1971) and present in high copy numbers in the genome (Prokopowich et al., 2003). The assay was designed to include all nucleotide variation within the target region and included a redundancy in the forward primer, to allow for the detection of all known canine *Babesia* spp. variants. It was possible to design genus-specific primers enclosing a region of species-specific variation, so the assay consisted of a single pair of primers that amplified a 127 nt region of *Babesia* 18S, with probes that were specific for each canine *Babesia* species.

Costa-Júnior et al., (2012) described the development of a real-time PCR assay targeting the internal transcribed spacer to detect *B. rossi*, *B. vogeli* and *B. canis*, which consisted on five primers and four probes. The assay was only tested on *B. vogeli* samples and was not validated.

Probes were optimised using 6-FAM and NED™ dyes, but in our experience, the NED™ dye is not detected efficiently by the StepOnePlus Real-Time PCR System, evidenced by shallow amplification curves and low peak amplification ΔRn. This probably resulted in a lower sensitivity for *B. vogeli* (NED™ probe), compared to using the probe labelled with another dye, which was not possible, as there are only three detection channels on the StepOnePlus Real-Time PCR System. The *B. rossi* probe is detected on one channel, the Xeno assay is available commercially only with VIC™ dye and is detected on the second channel.

PCR-based assays targeting the 18S gene have the potential to be very sensitive and specific. Souza et al., (2016) compared four different assays which targeted the 18S rRNA gene to diagnose *B. microti.* The assays included nested PCR, TaqMan and SYBR green chemistries. The SYBR green assay (Bloch *et al.*, 2013) was the most sensitive (but not specific), detecting 2.4 parasites/µl, which was comparable to that of nested PCR (Persing *et al*., 1992) at 2.4 parasites/µl. The TaqMan® assay (Hojgaard *et al*., 2014) had a detection limit of 12

parasites/µl although the assay detected samples with fewer parasites more frequently. It was not detailed how the detection limit was calculated and if the numbers represent an end-point detection, but in comparison, the 95% LOD in this study was the equivalent of 140 *B. rossi* parasites/µl and 593 *B. vogeli* parasites/µl blood. The 10% LOD in this study was the equivalent of 0.81 *B. rossi* parasites/µl and 81.82 *B. vogeli* parasites/µl blood, which compares favourably with the study of Souza et al., (2016), if what they reported was an end-point detection limit.

Bhoora et al., (2010a 2018) designed real-time PCR assays to target a region of the *B. caballi* and *Theileria* equi 18S rRNA genes and reported a 95% LOD between 1.4×10^{-4} and 2.8×10^{-4}% PE. The assay designed to detect *B. rossi* and *B. vogeli* in this study had a 95% LOD of between 2.1×10^{-3} and 9.3×10^{-3}% PE, and was 8–79 times less sensitive than the assays reported by Bhoora et al., (2010a 2018). Reasons for the differences in sensitivities between the assays may be attributed possibly to differences in the accuracy of measurement of the plasmid concentration by spectrophotometry, or the use of NED™ dye, which we believe is not detected efficiently by the StepOnePlus Real-Time PCR System.

Field samples were collected from dogs with clinical signs of babesiosis and the infection confirmed by visualisation of large *Babesia* parasites in a thin, stained capillary blood smear. *B. rossi* and *B. vogeli* cannot be distinguished morphologically by light microscopy.

The PCR was used to test 121 *Babesia* spp. positive field samples and five false negatives results were obtained. All the false negative results were positive by the PCR assay, but the C_T values of four of the samples identified as *B. rossi*, and one of the samples identified as *B. vogeli* by the RLB assay were above the 95% LOD C_T values and were therefore classified as negative. Out of the 86 negative field samples, 15 samples tested positive by the PCR assay (17.4%) but with C_T values above the 95% LOD C_T value and were therefore also classified as negative. It is not clear whether these results indicated subclinical infections in the negative control dog samples, or laboratory contamination in the PCR assay.

The PCR assay was compared to the RLB assay and two false-negatives results were obtained for the *B. rossi* assay and one false-negative result for the *B. vogeli* assay. All the false-negative results were positive by the PCR assay, but the C_T values were above the 95% LOD C_T values and were therefore classified as negative.

Due to the small sample size obtained for *B. vogeli* infections (n = 8), the sensitivity and specificity for the detection of this parasite is most likely inaccurate, as is evident by the wide CI (47.4–99.7) of the sensitivity calculation. More samples are needed to characterize the PCR assay for *B. vogeli* more accurately.

5. Conclusion

The 18S multiplex *B. rossi* and *B. vogeli* TaqMan® real-time PCR assay is a rapid, sensitive and specific method for detecting canine babesiosis and discriminating between *B. rossi* and

B. vogeli. This assay will be able to accurately and rapidly confirm babesiosis in canines and allow for treatment to be administered in the early stages of the disease, speeding up the recovery time in affected dogs.

Acknowledgments

Funding: This work was supported by the National Research Foundation under a grant held by Prof A.L. Leisewitz (CPRR13080726333), as well as by the Test Development Fund of the Department of Veterinary Tropical Diseases, University of Pretoria.

Appendix A

Customized protocol for the MagMax™ Express Particle Processor (Life Technologies)

[PROTOCOL PROPERTIES]

Name = AM1830_Mod3
Protocol template version = 2.6.0
Instrument type = KingFisher
Creator = mquan
Created = 30/9/2009 9:31:36
Description = RNA isolation from whole blood.
Kit = MagMAX-96 viral RNA Isolation Kit , AM1836
Plate layouts = Default

[PLATE LAYOUTS]

_____
Default

Plate type = KingFisher plate 200 ul
Plate change message = Change Default

A:
- volume = 20, name = Bead Mix (10 ul RNA binding beads, 10 ul Lysis/Binding Enhancer)
- volume = 50, name = Whole blood
- volume = 130, name = 65 ul Lysis/Binding Solution, 1 ul Carrier RNA, 2 ul Xeno RNA, 65 ul Isopropanol
B:
- volume = 150, name = Wash Solution 1
C:
- volume = 150, name = Wash Solution 1
D:
- volume = 150, name = Wash Solution 2
E:
- volume = 150, name = Wash Solution 2
F:
- volume = 50, name = Elution Buffer
G:
- EMPTY
H:

- EMPTY

[STEPS]

BIND
Step parameters
- Name = Lysis Binding 5 min
- Well = A, Default

Beginning of step:
- No Action = Yes

Bind parameters:
- Bind time = 5min 0s, speed = Fast dual mix

End of step:
- Collect beads = Yes, count = 5

WASH
Step parameters
- Name = 1st Wash I 1 min
- Well = B, Default

Beginning of step:
- Release = Yes, time = 0s, speed = Fast

Wash parameters:
- Wash time = 1min 0s, speed = Fast

End of step:
- Collect beads = Yes, count = 3

WASH
Step parameters
- Name = 2nd Wash I 1 min
- Well = C, Default

Beginning of step:
- Release = Yes, time = 0s, speed = Fast

Wash parameters:
- Wash time = 1min 0s, speed = Fast

End of step:
- Well = E, Default

Beginning of step:
- Release = Yes, time = 0s, speed = Fast

Wash parameters:
- Wash time = 1min 0s, speed = Fast

End of step:
- Collect beads = Yes, count = 2

_____

DRY
Step parameters
- Name = Dry 1 min
- Well = E, Default
- Dry time = 1min 0s
- Tip position = Outside well

_____

ELUTION
Step parameters
- Name = Elution 3 min
- Well = F, Default

Beginning of step:
- Release = Yes, time = 0s, speed = Fast

Elution parameters:
- Elution time = 3min 0s, speed = Bottom medium

Pause parameters:
- Pause for manual handling = No

Remove beads:
- Remove beads = Yes, collect count = 5, disposal well = B

Appendix B

Table B. Comparison of testing field samples by the reverse line blot (RLB) and PCR assays. Dogs were confirmed to be infected (Infection status) with large *Babesia* parasites by clinical signs of babesiosis and visualisation of thin, stained capillary blood smears. A 95% limit of detection cycle threshold (C_T) of 36.1177 for *B. rossi* and 37.8495 for *B. vogeli* was used.

Laboratory number	Infection status	RLB result	*B rossi* C_T	*B. vogeli* C_T
RLB 17/034	Positive	*B. rossi*	15.775	Undetermined
RE 15/566	Positive	*B. rossi*	17.508	Undetermined
RLB 17/052	Positive	*B. rossi*	17.612	Undetermined
RLB 17/028	Positive	*B. rossi*	17.834	Undetermined
RE 16/031	Positive	*B. rossi*	17.892	Undetermined

Laboratory number	Infection status	RLB result	*B rossi* C_T	*B. vogeli* C_T
RE 16/032	Positive	*B. rossi*	18.141	Undetermined
RE 16/017	Positive	*B. rossi*	18.160	Undetermined
RLB 17/032	Positive	*B. rossi*	18.350	Undetermined
RE 16/061	Positive	*B. rossi*	18.605	Undetermined
RE 16/020	Positive	*B. rossi*	20.053	Undetermined
RLB 17/031	Positive	*B. rossi*	20.198	Undetermined
RE 15/559	Positive	*B. rossi*	20.213	Undetermined
RLB 17/029	Positive	*B. rossi*	20.472	Undetermined
RE 16/034	Positive	*B. rossi*	20.487	Undetermined
RE 16/027	Positive	*B. rossi*	20.551	Undetermined
RLB 17/059	Positive	*B. rossi*	20.593	Undetermined
RE 16/036	Positive	*B. rossi*	20.767	Undetermined
RE17/125	Positive	*B. rossi*	20.842	Undetermined
RLB 17/055	Positive	*B. rossi*	20.922	Undetermined
RLB 17/038	Positive	*B. rossi*	20.982	Undetermined
RLB 17/036	Positive	*B. rossi*	20.999	Undetermined
RE 15/560	Positive	*B. rossi*	21.000	Undetermined
RE 15/563	Positive	*B. rossi*	21.211	39.994
RE 15/558	Positive	*B. rossi*	21.224	Undetermined
RE17/142	Positive	*B. rossi*	21.319	Undetermined
RE 16/013	Positive	*B. rossi*	21.452	Undetermined
RE 15/548	Positive	*B. rossi*	21.596	Undetermined

Laboratory number	Infection status	RLB result	*B rossi* C_T	*B. vogeli* C_T
RE 16/026	Positive	*B. rossi*	21.852	Undetermined
RE 15/549	Positive	*B. rossi*	21.922	Undetermined
RE 16/012	Positive	*B. rossi*	21.967	Undetermined
RE 16/021	Positive	*B. rossi*	22.174	Undetermined
RLB 17/053	Positive	*B. rossi*	22.289	Undetermined
RLB 17/030	Positive	*B. rossi*	22.563	Undetermined
RE17/144	Positive	*B. rossi*	22.727	Undetermined
RLB 17/033	Positive	*B. rossi*	22.809	Undetermined
RE17/124	Positive	*B. rossi*	22.823	Undetermined
RE 16/022	Positive	*B. rossi*	23.000	Undetermined
RE 16/019	Positive	*B. rossi*	23.057	Undetermined
RE17/159	Positive	*B. rossi*	23.097	Undetermined
RLB 17/037	Positive	*B. rossi*	23.199	Undetermined
RLB 17/035	Positive	*B. rossi*	23.231	Undetermined
RE17/132	Positive	*B. rossi*	23.304	Undetermined
RE 16/035	Positive	*B. rossi*	23.645	Undetermined
RE 15/550	Positive	*B. rossi*	23.949	Undetermined
RLB 17/056	Positive	*B. rossi*	23.987	Undetermined
RE 16/063	Positive	*B. rossi*	24.008	Undetermined
RE 16/014	Positive	*B. rossi*	24.092	Undetermined
RE 16/024	Positive	*B. rossi*	24.183	Undetermined
RE 16/033	Positive	*B. rossi*	24.273	Undetermined

Laboratory number	Infection status	RLB result	*B rossi* C_T	*B. vogeli* C_T
RE 16/023	Positive	*B. rossi*	24.378	Undetermined
RE17/154	Positive	*B. rossi*	24.388	Undetermined
RE 16/029	Positive	*B. rossi*	24.523	Undetermined
RE17/135	Positive	*B. rossi*	24.624	Undetermined
RE 16/015	Positive	*B. rossi*	24.624	Undetermined
RE 16/025	Positive	*B. rossi*	24.682	Undetermined
RE 15/554	Positive	*B. rossi*	24.889	Undetermined
RLB 17/027	Positive	*B. rossi*	25.025	Undetermined
RE17/143	Positive	*B. rossi*	25.076	Undetermined
RE 16/018	Positive	*B. rossi*	25.188	Undetermined
RE 15/547	Positive	*B. rossi*	25.258	Undetermined
RE17/126	Positive	*B. rossi*	25.661	Undetermined
RE17/139	Positive	*B. rossi*	25.661	Undetermined
RE17/162	Positive	*B. rossi*	25.805	Undetermined
RE17/146	Positive	*B. rossi*	25.829	Undetermined
RE17/168	Positive	*B. rossi*	25.842	Undetermined
RE 16/011	Positive	*B. rossi*	25.947	Undetermined
RE17/141	Positive	*B. rossi*	26.015	Undetermined
RE17/133	Positive	*B. rossi*	26.238	Undetermined
RE17/121	Positive	*B. rossi*	26.267	Undetermined
RE 15/562	Positive	*B. rossi*	26.328	Undetermined
RE 16/062	Positive	*B. rossi*	26.504	Undetermined

Laboratory number	Infection status	RLB result	*B rossi* C_T	*B. vogeli* C_T
RE 16/010	Positive	*B. rossi*	26.606	Undetermined
RE17/137	Positive	*B. rossi*	26.976	Undetermined
RE 15/565	Positive	*B. rossi*	27.005	Undetermined
RE17/130	Positive	*B. rossi*	27.124	Undetermined
RE17/167	Positive	*B. rossi*	27.161	Undetermined
RE17/153	Positive	*B. rossi*	27.276	Undetermined
RE 15/546	Positive	*B. rossi*	27.446	Undetermined
RE 16/030	Positive	*B. rossi*	27.454	Undetermined
RE17/147	Positive	*B. rossi*	27.593	Undetermined
RE 15/553	Positive	*B. rossi*	27.594	Undetermined
RE17/157	Positive	*B. rossi*	27.702	Undetermined
RE17/148	Positive	*B. rossi*	27.769	Undetermined
RE 15/564	Positive	*B. rossi*	27.771	Undetermined
RE17/164	Positive	*B. rossi*	28.133	Undetermined
RE17/131	Positive	*B. rossi*	28.187	Undetermined
RE17/163	Positive	*B. rossi*	28.280	Undetermined
RE 15/567	Positive	*B. rossi*	28.804	Undetermined
RE 15/552	Positive	*B. rossi*	28.859	Undetermined
RE17/138	Positive	*B. rossi*	28.859	Undetermined
RE 15/557	Positive	*B. rossi*	28.879	Undetermined
RE 15/556	Positive	*B. rossi*	28.885	Undetermined
RE17/123	Positive	*B. rossi*	29.039	Undetermined

Laboratory number	Infection status	RLB result	*B rossi* C_T	*B. vogeli* C_T
RE17/129	Positive	*B. rossi*	29.096	Undetermined
RE17/156	Positive	*B. rossi*	29.132	Undetermined
RE17/160	Positive	*B. rossi*	30.051	Undetermined
RE 16/028	Positive	*B. rossi*	30.329	Undetermined
RE17/122	Positive	*B. rossi*	30.345	Undetermined
RE17/171	Positive	*B. rossi*	30.861	Undetermined
RE17/172	Positive	*B. rossi*	31.033	Undetermined
RE17/155	Positive	*B. rossi*	31.039	Undetermined
RE17/140	Positive	*B. rossi*	31.334	Undetermined
RE17/149	Positive	*B. rossi*	31.514	Undetermined
RE17/158	Positive	*B. rossi*	31.678	Undetermined
RE 15/555	Positive	*B. rossi*	32.018	Undetermined
RE17/170	Positive	*B. rossi*	32.709	Undetermined
RE17/166	Positive	*B. rossi*	32.901	Undetermined
RE17/161	Positive	*B. rossi*	33.612	Undetermined
RE 15/561	Positive	*B. rossi*	35.244	Undetermined
RLB 16/010	Positive	*B. rossi*	36.398	Undetermined
RE17/169	Positive	*B. rossi*	37.337	Undetermined
RE 16/105	Positive	*B. vogeli*	Undetermined	28.131
RLB 17/054	Positive	*B. vogeli*	Undetermined	30.246
RE 15/253	Positive	*B. vogeli*	Undetermined	30.731
RE 16/119	Positive	*B. vogeli*	36.769	31.779

Laboratory number	Infection status	RLB result	*B rossi* C_T	*B. vogeli* C_T
RE 16/106	Positive	*B. vogeli*	Undetermined	32.490
RE 16/120	Positive	*B. vogeli*	Undetermined	33.399
RE 16/121	Positive	*B. vogeli*	Undetermined	33.637
RE 16/080	Positive	*B. vogeli*	Undetermined	38.493
RE 16/009	Positive	Negative	36.801	Undetermined
RE 16/052	Positive	Negative	37.515	Undetermined
RE 16/043	Negative	Negative	36.242	Undetermined
RLB 17/041	Negative	Negative	37.381	Undetermined
RE 16/115	Negative	Negative	37.464	Undetermined
RLB 17/051	Negative	Negative	37.476	Undetermined
RE 16/050	Negative	Negative	37.575	Undetermined
RE 16/048	Negative	Negative	37.619	Undetermined
RE 16/056	Negative	Negative	37.680	Undetermined
RE 16/055	Negative	Negative	38.078	Undetermined
RE 16/078	Negative	Negative	38.292	Undetermined
RLB 17/040	Negative	Negative	38.729	Undetermined
RLB 17/049	Negative	Negative	38.741	Undetermined
RE17/152	Negative	Negative	39.128	Undetermined
RE17/113	Negative	Negative	39.253	Undetermined
RE 16/041	Negative	Negative	39.512	Undetermined
RE17/173	Negative	Negative	39.809	Undetermined
RE 16/044	Negative	Negative	Undetermined	Undetermined

Laboratory number	Infection status	RLB result	*B rossi* C_T	*B. vogeli* C_T
RE 15/551	Negative	Negative	Undetermined	Undetermined
RE 16/001	Negative	Negative	Undetermined	Undetermined
RE 16/002	Negative	Negative	Undetermined	Undetermined
RE 16/003	Negative	Negative	Undetermined	Undetermined
RE 16/004	Negative	Negative	Undetermined	Undetermined
RE 16/005	Negative	Negative	Undetermined	Undetermined
RE 16/006	Negative	Negative	Undetermined	Undetermined
RE 16/007	Negative	Negative	Undetermined	Undetermined
RE 16/008	Negative	Negative	Undetermined	Undetermined
RE 16/039	Negative	Negative	Undetermined	Undetermined
RE 16/040	Negative	Negative	Undetermined	Undetermined
RE 16/042	Negative	Negative	Undetermined	Undetermined
RE 16/045	Negative	Negative	Undetermined	Undetermined
RE 16/046	Negative	Negative	Undetermined	Undetermined
RE 16/049	Negative	Negative	Undetermined	Undetermined
RE 16/051	Negative	Negative	Undetermined	Undetermined
RE 16/053	Negative	Negative	Undetermined	Undetermined
RE 16/054	Negative	Negative	Undetermined	Undetermined
RE 16/057	Negative	Negative	Undetermined	Undetermined
RE 16/058	Negative	Negative	Undetermined	Undetermined
RE 16/059	Negative	Negative	Undetermined	Undetermined
RE 16/065	Negative	Negative	Undetermined	Undetermined

Laboratory number	Infection status	RLB result	*B rossi* C_T	*B. vogeli* C_T
RE 16/066	Negative	Negative	Undetermined	Undetermined
RE 16/067	Negative	Negative	Undetermined	Undetermined
RE 16/068	Negative	Negative	Undetermined	Undetermined
RE 16/069	Negative	Negative	Undetermined	Undetermined
RE 16/071	Negative	Negative	Undetermined	Undetermined
RE 16/075	Negative	Negative	Undetermined	Undetermined
RE 16/076	Negative	Negative	Undetermined	Undetermined
RE 16/077	Negative	Negative	Undetermined	Undetermined
RE 16/079	Negative	Negative	Undetermined	Undetermined
RE 16/081	Negative	Negative	Undetermined	Undetermined
RE 16/082	Negative	Negative	Undetermined	Undetermined
RE 16/084	Negative	Negative	Undetermined	Undetermined
RE 16/087	Negative	Negative	Undetermined	Undetermined
RE 16/088	Negative	Negative	Undetermined	Undetermined
RE 16/090	Negative	Negative	Undetermined	Undetermined
RE 16/092	Negative	Negative	Undetermined	Undetermined
RE 16/093	Negative	Negative	Undetermined	Undetermined
RE 16/094	Negative	Negative	Undetermined	Undetermined
RE 16/096	Negative	Negative	Undetermined	Undetermined
RE 16/098	Negative	Negative	Undetermined	Undetermined
RE 16/099	Negative	Negative	Undetermined	Undetermined
RE 16/101	Negative	Negative	Undetermined	Undetermined

Laboratory number	Infection status	RLB result	*B rossi* C_T	*B. vogeli* C_T
RE 16/102	Negative	Negative	Undetermined	Undetermined
RE 16/104	Negative	Negative	Undetermined	Undetermined
RE 16/107	Negative	Negative	Undetermined	Undetermined
RE 16/108	Negative	Negative	Undetermined	Undetermined
RE 16/110	Negative	Negative	Undetermined	Undetermined
RE 16/112	Negative	Negative	Undetermined	Undetermined
RE 16/113	Negative	Negative	Undetermined	Undetermined
RE 16/114	Negative	Negative	Undetermined	Undetermined
RE 16/116	Negative	Negative	Undetermined	Undetermined
RE 16/117	Negative	Negative	Undetermined	Undetermined
RE 16/122	Negative	Negative	Undetermined	Undetermined
RE 16/123	Negative	Negative	Undetermined	Undetermined
RE17/111	Negative	Negative	Undetermined	Undetermined
RE17/115	Negative	Negative	Undetermined	Undetermined
RE17/116	Negative	Negative	Undetermined	Undetermined
RE17/117	Negative	Negative	Undetermined	Undetermined
RE17/118	Negative	Negative	Undetermined	Undetermined
RE17/120	Negative	Negative	Undetermined	Undetermined
RE17/150	Negative	Negative	Undetermined	Undetermined
RE17/151	Negative	Negative	Undetermined	Undetermined
RE17/174	Negative	Negative	Undetermined	Undetermined
RLB 17/039	Negative	Negative	Undetermined	Undetermined

Laboratory number	Infection status	RLB result	*B rossi* C_T	*B. vogeli* C_T
RLB 17/042	Negative	Negative	Undetermined	Undetermined
RLB 17/043	Negative	Negative	Undetermined	Undetermined
RLB 17/050	Negative	Negative	Undetermined	Undetermined
RE 16/064	Negative	Negative	Undetermined	Undetermined

References

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J. 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403-410. https://doi.org/10.1016/S0022-2836(05)80360-2.

Annoscia, G., Latrofa, M.S., Cantacessi, C., Olivieri, E., Manfredi, M.T., Dantas-Torres, F., Otranto, D., 2017. A new PCR assay for the detection and differentiation of *Babesia canis* and *Babesia vogeli*. Ticks Tick-borne Dis. 8(6), 862-865. https://doi.org/10.1016/j.ttbdis.2017.07.002.

Ano, H., Makimura, S., Harasawa, R., 2001. Detection of *Babesia* species from infected dog blood by polymerase chain reaction. J. Vet. Med. Sci. 63(1), 111-113. https://doi.org/10.1292/jvms.63.111.

Babes, V., 1888. Sur l'hemoglobinurie bacterienne du boeuf. CR Acad. Sci. 107:692-694.

Bekker, C.P., de Vos, S., Taoufik, A., Sparagano, O.A., Jongejan, F., 2002. Simultaneous detection of *Anaplasma* and *Ehrlichia* species in ruminants and detection of *Ehrlichia ruminantium* in *Amblyomma variegatum* ticks by reverse line blot hybridization. Vet. Microbiol. 89, 223-238. https://doi.org/10.1016/S0378-1135(02)00179-7.

Benson, D.A., Cavanaugh, M., Clark, K., Karsch-Mizrachi, I., Lipman, D.J., Ostell, J., Sayers, E.W. 2013. GenBank. Nucleic Acids Res. 41(D1), D36-42. https://doi: 10.1093/nar/gks1195.

Bhoora, R., Quan, M., Franssen, L., Butler, C.M., Van der Kolk, J.H., Guthrie, A.J., Zweygarth, E., Jongejan, F., Collins, N.E., 2010. Development and evaluation of real-time PCR assays for the quantitative detection of *Babesia caballi* and *Theileria equi* infections in horses from South Africa. Vet. Parasitol. 168, 201-211. https://doi.org/10.1016/j.vetpar.2009.11.011.

Bhoora, R.V., Pienaar, R., Cornelius, F., Josemans, A., Matthee, O., Marumo, R., Troskie, C., Mans, B.J., 2018. Multiplex hydrolysis-probe assay for the simultaneous detection of *Theileria equi* and *Babesia caballi* infections in equids. Vet. Parasitol. 255, 61-68. https://doi.org/10.1016/j.vetpar.2018.03.022.

Birkenheuer, A.J., Levy, M.G., Breitschwerdt, E.B., 2003. Development and evaluation of a seminested PCR for detection and differentiation of *Babesia gibsoni* (Asian genotype) and *B. canis* DNA in canine blood samples. J. Clin. Microbiol. 41, 4172-4177. https://doi: 10.1128/JCM.41.9.4172-4177.2003.

Birkenheuer, A.J., Neel, J., Ruslander, D., Levy, M.G., Breitschwerdt, E.B., 2004. Detection and molecular characterization of a novel large *Babesia* species in a dog. Vet. Parasitol. 124, 151-160. https://doi.org/10.1016/j.vetpar.2004.07.008.

Birnstiel, M.L., Chipchase, M., Speirs, J., 1971. The ribosomal RNA cistrons. In Progress in nucleic acid research and molecular biology. 11, 351-389. Academic Press. https://doi: 10.1139/G02-103.

Bloch, E.M., Lee, T.H., Krause, P.J., Telford III, S.R., Montalvo, L., Chafets, D., Usmani-Brown, S., Lepore, T.J., Busch, M.P., 2013. Development of a real-time polymerase chain reaction assay for sensitive detection and quantitation of *Babesia microti* infection. Transfusion. 53, 2299-2306. https://doi.org/10.1111/trf.12098.

Böse, R., Jorgensen, W.K., Dalgliesh, R.J., Friedhoff, K.T., De Vos, A.J., 1995. Current state and future trends in the diagnosis of babesiosis. Vet. Parasitol. 57, 61-74. https://doi.org/10.1016/0304-4017(94)03111-9.

Carret, C., Walas, F., Carcy, B., Grande, N., Précigout, É., Moubri, K., Schetters, T.P., Gorenflot, A., 1999. *Babesia canis canis, Babesia canis vogeli, Babesia canis rossi*: differentiation of the three subspecies by a restriction fragment length polymorphism analysis on amplified small subunit ribosomal RNA genes. J. Eukaryotic Microbiol. 46, 298-301. https://doi.org/10.1111/j.1550-7408.1999.tb05128.x.

Collett, M.G., 2000. Survey of canine babesiosis in South Africa. J. S. Afr. Vet. Ass. 71, 180-186.

Conrad, P., Thomford, J., Yamane, I., Whiting, J., Bosma, L., Uno, T., Holshuh, H.J., Shelly, S., 1991. Hemolytic anemia caused by *Babesia gibsoni* infection in dogs. J. Am. Vet. Med. Assoc. 199(5), 601-605.

Costa-Júnior, L.M., Zahler-Rinder, M., Ribeiro, M.F.B., Rembeck, K., Rabelo, E.M.L., Pfister, K., Passos, L.M.F., 2012. Use of a Real Time PCR for detecting subspecies of *Babesia canis*. Vet. Parisitol. 188, 160-163. https://doi.org/10.1016/j.vetpar.2012.03.015.

Dalrymple, B.P., Dimmock, C.M., Parrodi, F., Wright, I.G., 1992. *Babesia bovis*, *Babesia bigemina*, *Babesia canis*, *Babesia microti* and *Babesia rodhaini*: comparison of ribosomal RNA gene organization. Int. J. Parasitol. 22(6), 851-855. https://doi.org/10.1016/0020-7519(92)90139-C.

Dantas-Torres, F., 2008. The brown dog tick, *Rhipicephalus sanguineus* (Latreille, 1806) (Acari: Ixodidae): from taxonomy to control. Vet. Parasitol. 152(3-4), 173-185. https://doi.org/10.1016/j.vetpar.2007.12.030.

Duarte, S.C., Linhares, G.F.C., Romanowsky, T.N., da Silveira Neto, O.J., Borges, L.M.F., 2008. Assessment of primers designed for the subspecies-specific discrimination among *Babesia canis canis*, *Babesia canis vogeli* and *Babesia canis rossi* by PCR assay. Vet. Parasitol. 152, 16-20. https://doi.org/10.1016/j.vetpar.2007.12.013.

Földvári, G., Hell, E., Farkas, R., 2005. *Babesia canis canis* in dogs from Hungary: detection by PCR and sequencing. Vet. Parasitol. 127, 221-226. https://doi.org/10.1016/j.vetpar.2004.10.016.

García, A.T.C., 2006. Piroplasma infection in dogs in northern Spain. Vet. Parasitol. 138, 97-102. https://doi.org/10.1016/j.vetpar.2006.01.043.

Gubbels, J.M., De Vos, A.P., Van Der Weide, M., Viseras, J., Schouls, L.M., De Vries, E., Jongejan, F., 1999. Simultaneous detection of bovine *Theileria* and *Babesia* species by Reverse Line Blot Hybridization. J. Clin. Microbiol. 37, 1782-1789.

Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series 41, 95-98.

Heid, C.A., Stevens, J., Livak, K.J., Williams, P.M., 1996. Real time quantitative PCR. Genome Res. 6, 986-994. https://doi.10.1101/gr.6.10.986.

Higuchi, R., Fockler, C., Dollinger, G., Watson, R., 1993. Kinetic PCR analysis: real-time monitoring of DNA amplification reactions. Nat. Biotechnol. 11, 1026. https://doi.org/10.1038/nbt0993-1026.

Hojgaard, A., Lukacik, G., Piesman, J., 2014. Detection of *Borrelia burgdorferi, Anaplasma phagocytophilum* and *Babesia microti*, with two different multiplex PCR assays. Ticks Tick-borne Dis. 5, 349-351. https://doi.org/10.1016/j.ttbdis.2013.12.001.

Homer, M.J., Aguilar-Delfin, I., Telford, S.R., Krause, P.J., Persing, D.H., 2000. Babesiosis. Clin. Microbiol. Rev. 13, 451-469. https://doi.10.1128/CMR.13.3.451-469.2000.

Horak, I.G., 1995. Ixodid ticks collected at the Faculty of Veterinary Science, Onderstepoort, from dogs diagnosed with *Babesia canis* infection. J. S. Afr. Vet. Ass. 66, 170-171.

Jacobson, L.S., Clark, I.A., 1994. The pathophysiology of canine babesiosis: new approaches to an old puzzle. J. S. Afr. vet. Ass. 65, 134.

Kaltenboeck, B., Wang, C., 2005. Advances in real-time PCR: application to clinical laboratory diagnostics. Adv. Clin Chem. 40(4):219-259.

Katoh, K., Misawa, K., Kuma, K., Miyata, T. 2002. MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. Nucleic Acids Res. 30, 3059–3066. https://doi.org/10.1093/nar/gkf436.

Katoh, K. 2013. MAFFT version 7. https://mafft.cbrc.jp/alignment/software/ (accessed 15 March 2016).

Kjemtrup, A.M., Kocan, A.A., Whitworth, L., Meinkoth, J., Birkenheuer, A.J., Cummings, J., Boudreaux, M.K., Stockham, S.L., Irizarry-Rovira, A., Conrad, P.A., 2000. There are at least three genetically distinct small piroplasms from dogs. Int. J. Parasitol. 30, 1501-1505. https://doi.org/10.1016/S0020-7519(00)00120-X.

Kjemtrup, A.M., Wainwright, K., Miller, M., Penzhorn, B.L., Carreno, R.A., 2006. *Babesia conradae*, sp. nov., a small canine *Babesia* identified in California. Vet. Parasitol. 138, 103-111. https://doi.org/10.1016/j.vetpar.2006.01.044.

Leisewitz, A.L., Jacobson, L.S., De Morais, H.S., Reyers, F., 2001. The mixed acid-base disturbances of severe canine babesiosis. J. Vet. Int. Med. 15, 445-452. https://doi.org/10.1111/j.1939-1676.2001.tb01573.x.

Levine, N.D., 1988. The Protozoa Phylum Apicomplexa. CRC Press, Boca Raton, Florida.

Loftis, A.D., Reeves, W.K., 2012. Principles of Real-Time PCR. Veterinary PCR Diagnostics. 3.

Matijatko, V., Kiš, I., Torti, M., Brkljačić, M., Kučer, N., Rafaj, R.B., Grden, D., Živičnjak, T., Mrljak, V., 2009. Septic shock in canine babesiosis. Vet. Parasitol. 162, 263-270. https://doi.org/10.1016/j.vetpar.2009.03.011.

Matjila, P.T., Penzhorn, B.L., Bekker, C.P.J., Nijhof, A.M., Jongejan, F., 2004. Confirmation of occurrence of *Babesia canis vogeli* in domestic dogs in South Africa. Vet. Parasitol. 122, 119-125. https://doi.org/10.1016/j.vetpar.2004.03.019.

Matjila, P.T., Leisewitz, A.L., Jongejan, F., Penzhorn, B.L., 2008. Molecular detection of tick-borne protozoal and ehrlichial infections in domestic dogs in South Africa. Vet. Parasitol. 155, 152-157. https://doi.org/10.1016/j.vetpar.2008.04.012.

Mullis, K., Faloona, F., Scharf, S., Saiki, R.K., Horn, G.T., Erlich, H., 1986. Specific enzymatic amplification of DNA in vitro: the polymerase chain reaction. In *Cold Spring Harbor symposia on quantitative biology.* Cold Spring Harbor Laboratory Press. 51: 263-273. https://doi:10.1101/SQB.1986.051.01.032.

Nagore, D., Garcıa-Sanmartın, J., Garcıa-Pérez, A.L., Juste, R.A., Hurtado, A., 2004. Detection and identification of equine *Theileria* and *Babesia* species by reverse line blotting: epidemiological survey and phylogenetic analysis. Vet. Parasitol. 123, 41-54. https://doi.org/10.1016/j.vetpar.2004.04.010.

NCBI, 2017a. GenBank https://www.ncbi.nlm.nih.gov/genbank (accessed 15 March 2016).

NCBI, 2017b. BLAST https://blast.ncbi.nlm.nih.gov/Blast.cgi (accessed 15 March 2016).

Nuttall, G.H., 1910. On haemoprotozoa occurring in wild animals in Africa, I. *Piroplasma rossi* n. sp. and *Haemogregarina canis adusti* n. sp. found in the jackal. Parasitology. 5, 61. https://doi.org/10.1017/S0031182000001955.

Oyamada, M., Davoust, B., Boni, M., Dereure, J., Bucheton, B., Hammad, A., Itamoto, K., Okuda, M., Inokuma, H., 2005. Detection of *Babesia canis rossi, B. canis vogeli*, and *Hepatozoon canis* in dogs in a village of eastern Sudan by using a screening PCR and sequencing methodologies. Clin. Vaccine Immunol. 12, 1343-1346. https://doi: 10.1128/CDLI.12.11.1343-1346.2005.

Patton, W.S., 1910. Preliminary report on a new piroplasm (*Piroplasma gibsoni* sp. nov.) found in the blood of the hounds of the Madras Hunt and subsequently discovered in the blood of the jackal *Canis aureus*. Bull. Soc. Pathol. Exot. 3, 274-280.

Persing, D.H., Mathiesen, D., Marshall, W.F., Telford, S.R., Spielman, A., Thomford, J.W., Conrad, P.A., 1992. Detection of *Babesia microti* by polymerase chain reaction. J. Clin. Microbiol. 30, 2097-2103.

Piana, G.P., Galli-Valerio, B., 1895. Su di un'infezione del cane con parassiti endoglobulari nel sangue. Nota Prev Mod Zooiatro. 6, 163-169.

Prokopowich, C.D., Gregory, T.R., Crease, T.J., 2003. The correlation between rDNA copy number and genome size in eukaryotes. Genome. 46, 48-50.

Reichenow, E., 1937. Ueber die Entwicklung von Theileria parva, dem Erreger des Küstenfiebers der Rinder. *Rhipicephalus appendiculatus.* Zbl Bakt Parasitenk Inf (Erste Abt/Orig). 140, 223-226.

Schetters, T.P., Kleuskens, J.A.G.M., Van De Crommert, J., De Leeuw, P.W.J., Finizio, A.L., Gorenflot, A., 2009. Systemic inflammatory responses in dogs experimentally infected with *Babesia canis*; a haematological study. Vet. Parasitol. 162, 7-15. https://doi.org/10.1016/j.vetpar.2009.02.012.

Scurell, E. 2006. What is your diagnosis? J. Small Anim. Pract. 47, 226–227.

Solano-Gallego, L., Trotta, M., Carli, E., Carcy, B., Caldin, M., Furlanello, T., 2008. *Babesia canis canis* and *Babesia canis vogeli* clinicopathological findings and DNA detection by means of PCR-RFLP in blood from Italian dogs suspected of tick-borne disease. Vet. Parasitol. 157, 211-221. https://doi.org/10.1016/j.vetpar.2008.07.024.

Souza, S.S., Bishop, H.S., Sprinkle, P., Qvarnstrom, Y., 2016. Comparison of *Babesia microti* real-time polymerase chain reaction assays for confirmatory diagnosis of babesiosis. Am. J. Trop. Med. Hyg. 95, 1413-1416. https://doi.org/10.4269/ajtmh.16-0406.

[dataset] Troskie, M., de Villiers, L., Leisewitz, A., Oosthuizen, M.C., Quan, M. 2018. Development and analytical validation of a multiplex, real-time PCR assay for *Babesia rossi* and *B. vogeli*. Mendeley Data.

Uilenberg, G., Franssen, F.F.J., Perie, N.M., Spanjer, A.A.M., 1989. Three groups of *Babesia canis* distinguished and a proposal for nomenclature. Vet. Quarterly. 11, 33-40. https://doi.org/10.1080/01652176.1989.9694194.

Uilenberg, G., 2006. *Babesia* - a historical overview. Vet. Parasitol. 138, 3-10. https://doi.org/10.1016/j.vetpar.2006.01.035.

Van Heerden, J., Reyers, F., Stewart, C.G., 1983. Treatment and thrombocyte levels in experimentally induced canine ehrlichiosis and canine babesiosis. Onderstepoort J. Vet. Res. 50, 267-270.

Welzl, C., Leisewitz, A.L., Jacobson, L.S., Vaughan-Scott, T., Myburgh, E., 2001. Systemic inflammatory response syndrome and multiple-organ damage/dysfunction in complicated canine babesiosis. J. S. Afr. Vet. Assoc. 72, 158-162.

Wozniak, E.J., Barr, B.C., Thomford, J.W., Yamane, I., McDonough, S.P., Moore, P.F., Naydan, D., Robinson, T.W., Conrad, P.A., 1997. Clinical, anatomic, and immunopathologic characterization of *Babesia gibsoni* infection in the domestic dog (*Canis familiaris*). J. Parasitol. 83, 692-699.

Zahler, M., Schein, E., Rinder, H., Gothe, R., 1998. Characteristic genotypes discriminate between *Babesia canis* isolates of differing vector specificity and pathogenicity to dogs. Parasitol. Res. 84, 544-548.