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Original article

Development and validation of a TaqMan® probe- based real-time PCR assay for detection of *Ehrlichia canis*

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ABSTRACT

Ehrlichiosis is a potentially fatal zoonotic tick-borne disease, caused by a pleomorphic Gram-negative bacterium. It occurs worldwide and affects humans, domestic and wild animals. Dogs infected with *Ehrlichia canis* develop canine monocytic ehrlichiosis (CME), a significant infectious disease of canines. TaqMan® based real-time PCR assays to detect *Ehrlichia* spp. affecting dogs were developed and a real-time PCR assay specific for *E. canis* validated. The efficiency of the assay was 93% and the 95% limit of detection was 33 *E. canis* plasmid copies/μl of blood (95% confidence interval: 23 - 58). The assay was specific for *E. canis* when tested against other haemoparasites. Consistent repeatability was observed, with an inter-run standard deviation (SD) range between 0.33 and 1.29 and an intra-run SD range between 0.04 and 1.14. Field samples were tested in parallel by both the *E. canis* real-time PCR assay and a reverse line blot hybridization assay. The results were in agreement for the two assays, with an exception of two out of 121 samples. Bayesian latent class analysis was used to calculate a diagnostic sensitivity of the *E. canis* real-time PCR assay of 90% and a specificity of 92%. This assay is a sensitive and reliable molecular detection method for *E. canis* and will be a useful tool for early diagnosis and timely treatment for this haemoparasite.

1. Introduction

Ehrlichiosis is an important zoonotic tick-borne disease that affects dogs worldwide. It is caused by a Gram-negative bacterium in the order *Rickettsiales*, family *Anaplasmataceae* and genus *Ehrlichia/Anaplasma* (Dumler et al., 2001; Mylonakis et al., 2019; Mylonakis and Theodorou, 2017; Rikihisa, 1991). The genus *Ehrlichia* consists of several species, but only *Ehrlichia ewingii, Ehrlichia chaffeensis,* and *Ehrlichia canis* are known to affect dogs (Little, 2010; Walker and Dumler, 1996). Dogs infected with *E. canis* develop canine monocytic ehrlichiosis (CME), a significant infectious disease of canines.

Transmission of these pathogens occurs through a tick vector in the family Ixodidae and occasionally by medical procedures that involve blood transfusions, organ transplants, or bone marrow transplants (McQuiston et al., 2000). The spread of each pathogen is dependant on the availability of the vectors and mammalian reservoir hosts (Hinrichsen et al., 2001).

Canine monocytic ehrlichiosis presents in different phases; the acute, subclinical phases, or chronic with non-specific clinical signs (Buhles et al., 1974; Harrus et al., 1997; Rodríguez-Alarcón et al., 2020). The acute phase persists for 2–4 weeks and can include clinical signs such as fever, lethargy, depression, splenomegaly, anorexia, lymphadenopathy, weight loss, thrombocytopenia, anaemia, hypergammaglobulinaemia, pancytopenia, haemorrhages, epistaxis and/or vomiting (Fourie et al., 2015; Harrus, 2015). In subacute or chronic cases of CME, the organism may be present in the circulation at undetectable levels or be sequestered in tissue and the period varies from months to years (Harrus et al., 2004, 1998; Iqbal and Rikihisa, 1994). Not all dogs ever progress to the chronic stage but when they do, the prognosis is worse. Multiple infections with more than one rickettsial pathogen are common, due to shared vectors or concurrent exposure to multiple tick vectors (Kordick et al., 1999), which may complicate the diagnosis.

Various tests have been described to diagnose ehrlichiosis, such as polymerase chain reaction (PCR) assays, cell culture, serological tests including Western immunoblotting, enzyme-linked immunosorbent assay (ELISA), and indirect immunofluorescence assay (IFA).

Ehrlichia canis has been detected using a conventional PCR assay targeting the 16S rRNA gene where amplicons were detected with

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agarose gel electrophoresis (Iqbal et al., 1994). Another conventional PCR assay with biotin-streptavidin chemiluminescent hybridization detection of the 16S rRNA gene was reported as reliable, specific and its sensitivity exceeded that of cell culture isolation (Beck et al., 1989; McBride et al., 1996). A nested PCR assay has also been described to detect *Ehrlichia* spp.; however, chances of contamination which may lead to false-positive results are high when using this method, which is also more laborious and costly (Yabsley et al., 2008).

A reverse line blot (RLB) hybridization assay targeting the 16S rRNA gene with species-specific probes for the detection of *Anaplasma* and *Ehrlichia* species has been described (Bekker et al., 2002). Using an *E. canis* probe previously described by Schouls et al. (1999), this assay could detect *Ehrlichia ovina*; however, the sensitivity of the assay was unknown although all species could be detected by their relevant species-specific probes with no cross-reactivity (Bekker et al., 2002). Another RLB hybridization assay targeting the 18S rRNA for *Theileria* and *Babesia* which can co-exist with *Anaplasma* and *Ehrlichia* has been developed (Gubbels et al., 1999). A disadvantage of the RLB hybridization assay is that it is very laborious and time-consuming to perform, resulting in a slow turnaround time.

Thomson et al. (2018) published the first probe-based TaqMan® assay that applied an alternative genomic target *gltA* (citrate synthase) gene. This assay was more specific and faster to run compared to an isothermal PCR (PCRun®, Biogal ACS Galed Labs, Israel) targeting the 16S rRNA gene, but the sensitivity of the two assays yielded similar results. A one-step PCR assay targeting *gltA* was specific and sensitive, but this method required digestion of PCR product with *Hin*dIII enzyme before analysis on an agarose gel (Marsilio et al., 2006).

Published multiplex assays include a triplex real-time PCR (qPCR) assay to detect *E. chaffeensis, E. ewingii*, and *E. canis*, using genus-specific primers targeting the *dsb* (disulfide oxidoreductase) gene and species-specific TaqMan® probes (Doyle et al., 2005), a multiplex qPCR assay to detect *E. canis* 16S rRNA and *B. vogeli* heat shock protein 70 (Peleg et al., 2010) and a multiplex qPCR assay to detect the 16S rRNA genes of *E. chaffeensis, E. canis, Anaplasma phagocytophilum* and *Anaplasma platys* (Sirigireddy and Ganta, 2005).

Some of the tests mentioned above have various limitations, such as the lack of sensitivity and specificity or are laborious to perform, and require skilled personnel to analyse the results (Ndip et al., 2005; Sykes, 2014; Wen et al., 1997). On the other hand, PCR and sequencing are sensitive and specific methods for pathogen characterization (Iqbal et al., 1994).

There is a need to develop a rapid, sensitive, and specific diagnostic tool to detect *E. canis* in dogs for accurate, timely, and effective treatment to be implemented. This tool would reduce the hospitalization and recovery time in infected dogs, as well as the inappropriate use of antibiotics which could lead to antimicrobial resistance. The 16S gene is an ideal target for qPCR assay design as it is used for the classification of bacteria and consists of conserved and hypervariable regions (Clarridge, 2004; Wilson et al., 1988). The conserved regions were used to design group-specific primers and species-specific probes. This approach allows for the design of species-specific assays that can be multiplexed which provides an advantage over other published assays. In this study, we developed a real-time PCR assay with group-specific primers and species-specific probes and validated an assay to detect *E. canis*.

2. Materials and methods

2.1. Sample collection

Blood samples were obtained specifically for this study from dogs suspected to have ehrlichiosis and were presenting with these clinical signs, non-regenerative anaemia (pale mucous membranes, low haematocrit, and lack of regeneration on a peripheral blood smear), thrombocytopaenia on a peripheral blood smear, easily palpable lymph nodes, large spleen, chronic history of illness (sometimes with weight loss), epistaxis, uveitis, pyrexia, and leukopaenia. Samples were collected at the University of Pretoria's (UP) Onderstepoort Veterinary Academic Hospital (OVAH) ($n = 5$) and Mamelodi Animal Health Clinic (MAHC) ($n = 9$). Dog owners signed consent forms to allow the use of samples for research purposes. The dogs were restrained by the veterinarians working at the sampling sites and two to five millilitres (ml) of blood was collected into ethylenediaminetetraacetic acid (EDTA) tubes via jugular vein venipuncture. Biobank Whatman® Flinders Technology Associates card samples from Namibia ($n = 55$) submitted to the Department of Veterinary Tropical Diseases (DVTD) for diagnostic testing for haemoparasites were also used in this study (Appendix A).

This study obtained approval from the University of Pretoria's Animal Ethics Committee (project no. V099–17), and Section 20 clearance (ref. 12/11/1/1/6) from the Department of Agriculture, Land Reform and Rural Development (DALRRD).

2.2. TaqMan® MGB real-time PCR assay design

Ehrlichia canis 16S rRNA gene sequences were downloaded from GenBank® (www.ncbi.nlm.nih.gov/genbank) and aligned using the online version of MAFFT (https://mafft.cbrc.jp/alignment/server) (Katoh et al., 2017) using default settings. The *E. canis* strain Oklahoma GenBank® sequence (NR_118741) was used as a reference sequence. Sequence alignment analysis and editing was performed with BioEdit sequence alignment editor (Hall, 1999). Identical sequences were identified and collapsed with the DAMBE software (Xia, 2018).

A set of group-specific primers were designed along with species-specific TaqMan® minor groove binder (MGB) probes using Primer Express® version 3.0.1 (Applied Biosystems, USA) (Table 1). A probe was labelled with a fluorescent reporter dye at the 5′-end and a nonfluorescent quencher-minor groove binder (NFQ-MGB) at the 3′-end. To test for non-specific binding *in silico*, a nucleotide Basic Local Alignment Search Tool (BLASTn) screening was performed for both the primers and probe on the National Center for Biotechnology Information (NCBI) website (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.3. Primer and probe optimization

The primer concentration was optimized using a strong positive *E. canis* sample (RE16/084) by testing 100, 200, 400, and 800 nM primer in the PCR reaction in triplicate, with a constant probe concentration of 250 nM. Amplification curves were analysed visually and the lowest primer concentration that yielded a steep sigmoidal amplification curve with the lowest cycle threshold (C_T) was selected. The primer concentration was limited to allow for multiplexed assays. Similarly, different probe concentrations in the PCR reaction of 50, 100, 150, 200, and 250 nM were tested in triplicate, with a constant optimized primer concentration of 200 nM.

2.4. Construction of a plasmid positive control

A plasmid control was produced from a diagnostic sample (RE16/076), which tested positive for *E. canis* by RLB hybridization assay and the TaqMan® MGB real-time PCR assay. A set of PCR primers (Table 2) targeting a region between nucleotides 41 and 330 of the 16S gene were designed in PrimerQuest® online (Integrated DNA Technologies, Inc. USA) and synthesised by Integrated DNA Technologies (USA). Sequence JN622141 *E. canis* strain NGR clone 64 16S rRNA gene was used for the numbering of the nucleotides.

The PCR assay comprised approximately 75 ng (2 µl) of DNA template, Phusion Flash High-Fidelity PCR master mix containing Phusion Flash DNA polymerase, dNTP's, and $MgCl_2$ (Thermo Fisher Scientific, USA) in a final volume of 25 µl. Template amplification was performed on a Gene Amp® PCR systems 2700 (Applied Biosystems, USA) with the following conditions: initial denaturing at 98 °C for 10 s, followed by 30 cycles of 98 °C for 1 s, annealing at 55 °C for 5 s and extension 72 °C for

Table 1
Sequence and characteristics of the group-specific *Ehrlichia* forward / reverse primers (F/R) and *Ehrlichia* species-specific probe (P) targeting the 16S gene (V1).

Name	Start	Stop	Primer sequence (5′−3′)	Length	TM (°C)	% GC
AnapEhrlichia_F	23	44	AGCYTAACACATGCAAGTCGAA	22	59	45
AnapEhrlichia_R	82	103	TTACTCACCCGTCTGCCACTAA	22	58	50
E. canis_P	60	76	AGCCTCTGGCTATAGGA	17	69	53

Table 2
Plasmid construction primers designed to target nucleotides 41–330 bp of the *E. canis* 16S rRNA gene using JN622141 for numbering.

Name		Start	Stop	Primer sequence (5′−3′)	Length	TM (°C)	% GC
16S_F	Forward	41	62	TGCATGAGTCCAAGCCATAATG	22	59.6	45
16S_R	Reverse	302	330	TACGTTAGATTAGCTAGTTGGTGAGGTAA	29	58.3	38

15 s, with a final extension at 72 °C for 1 min.

The PCR product was purified using a High Pure PCR product purification kit (Roche, South Africa) and cloned into a pJET1.2/blunt cloning vector (Thermo Fisher Scientific, USA). Nine randomly picked colonies were amplified with pJET primers to check for the presence of the insert, confirmed by evaluation of the size of the amplicon on a 2% agarose gel. The amplicons were sequenced using the Sanger method (Inqaba Biotec™, South Africa) and analysed using CLC Genomic Workbench 7 software (Qiagen bioinformatics).

The competent high-efficiency *Escherichia coli* JM109 cells (Promega, USA) were used for vector transformation according to the manufacturer's instructions. ImMedia™ Amp liquid broth (Invitrogen, USA) was used to grow the culture at 37 °C for 1 h 30 min. Using standard procedure, the culture was plated on two imMedia™ Amp Blue (Invitrogen, USA) plates and incubated overnight at 37 °C.

Colony PCR screening for the correct DNA insert was performed on the colonies using a reaction mixture comprised of Dream *Taq* buffer (Thermo Fisher Scientific, USA), the pJET1.2_F primer (5′-CGA CTC ACT ATA GGGAGA 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. Amplification conditions were set as follows: initial denaturing at 95 °C for 3 min (1 cycle), followed by denaturing at 94 °C for 30 s (25 cycles), annealing at 60 °C for 30 s (25 cycles), and extension 72 °C for 1 min (1 cycle). Glycerol stocks for each clone were prepared by growing selected colonies overnight in imMedia™ Amp liquid broth (Invitrogen, USA) at 37 °C in a shaking incubator. Five hundred µl of the culture was mixed gently with 500 µl (50%) glycerol and stored at −80 °C.

Recombinant plasmid purification from overnight cultures was performed using a High Pure Plasmid Isolation Kit (Roche Diagnostics, Mannheim, Germany), by following the manufacturer's instructions. The eluted plasmid DNA concentration was determined by taking an average of multiple readings from BioTek™ PowerWave™ (Analytical and Diagnostic Products, South Africa), Qubit 2.0 fluorometer (Life Technologies; Carlsbad, CA, USA), and Trinean Xpose (Anatech Instruments, South Africa) spectrophotometers. It was recognized that the *E. canis* genome has one copy of each of the rRNA genes 5S, 16S, and 23S (Mavromatis et al., 2006). The copy number of the plasmid per µl was calculated using the formula: 6.022×10^{23} (copy number/mol) × concentration (g/µl) ÷ molar mass (g/mol). A small volume of the recombinant plasmid (5 µl) was sent to Inqaba Biotec™ (Pretoria, RSA) for Sanger sequencing, and the remaining plasmid was stored at −20 °C. Further analysis of the sequences was done using CLC Genomic Workbench 7 software (Qiagen bioinformatics). The NCBI BLAST search was used to identify the contigs.

2.5. Nucleic acid purification

Nucleic acid purification of blood/FTA samples was performed using the KingFisher™ Duo Prime Purification System and MagMAX™ CORE Nucleic Acid Purification Kit (ThermoFisher Scientific, USA). The simple workflow method as described by the manufacturer was used to purify nucleic acid from blood samples following instructions. Two hundred microlitres of blood sample was used as the starting volume and approximately 10 mm^2 of the FTA blood spot cut using a sterile scalpel blade was used for purification. A standard extraction programme (MagMAX_Core_Duo_No_heat.bdz) was used. The nucleic acid was eluted in 90 µl of MagMAX™ CORE Elution Buffer.

Two standard MagMAX™ CORE Nucleic Acid Purification Kit protocols, the complex workflow, and the digestion workflow were compared to purify nucleic acid from FTA card samples.

2.6. Real-time PCR

The components of the TaqMan® MGB real-time PCR assay consisted of 200 nM forward primer, 200 nM reverse primer, 250 nM probe (all final concentrations), 10 µl 2x TaqMan® Fast Advanced Master Mix (Thermo Fisher Scientific, USA), 2 µl sample template/plasmid (positive control) and distilled water to make up a reaction volume of 20 µl. This assay was performed on the StepOnePlus™ Real-Time PCR System (Applied Biosystems, USA) and consisted of a UNG incubation step at 50 °C for 2 min (1 cycle), followed by polymerase activation at 95 °C for 10 s (1 cycle), then denaturing at 95 °C for 1 s and annealing/extension at 60 °C for 20 s (40 cycles).

2.7. Laboratory validation

2.7.1. Assay linear range and efficiency

A full blood cell count was performed using an ADVIA 2120 hematology System (Siemens Healthcare GmbH, Germany) on the blood from a greyhound dog that served as a blood donor for the OVAH and was tested regularly for the presence of haemoparasites.

A ten-fold dilution series (from 10° to 10^{-10}) of plasmid control DNA was prepared with Tris-EDTA buffer as the diluent. Ten µl of each plasmid DNA dilution was added to 190 µl of the greyhound blood to make a dilution series of plasmid spiked canine blood.

2.7.2. Analytical sensitivity and variation

A starting dilution of 10^{-6} plasmid DNA was made to prepare a two-fold dilution series of blood spiked with $10^{1.43}$ to $10^{7.43}$ plasmid DNA, to cover the range from 0 to 100% pathogen detection. Five separate nucleic acid purifications were performed from each dilution. Five separate PCR runs were performed to test each purified dilution. The 95% limit of detection (LOD) was calculated by probit analysis (SPSS Statistics v25, IBM Analytics, USA).

2.7.3. Analytical specificity

Assay specificity was evaluated by testing diagnostic samples submitted to the DVTD, University of Pretoria that had been found to be positive for other pathogens by the RLB hybridization assay. Other

tested samples included blood vaccines purchased from Onderstepoort Biological Products, samples positive on microbiome sequence analysis by Kolo et al. (2020), and in-house constructed plasmids (Table 3).

2.7.4. Repeatability

Calculations of the intra-run and inter-run standard deviations (SD) and coefficient of variation (CV) were done with Microsoft Excel using results from analytical sensitivity and variation. The total coefficient of variation (CV) was calculated by the formula: CV = total SD / (mean C_T value of all replicates).

2.8. Diagnostic validation

The *Ehrlichia canis* TaqMan® MGB real-time PCR assay that was developed was tested on a total of 121 samples. There were two populations of samples tested. The first population were South African canine blood samples ($n = 52$), submitted to the DVTD biobank from OVAH for diagnostic testing for haemoparasites. Other blood samples were obtained directly for this study and included OVAH blood samples ($n = 5$) and MAHC blood samples ($n = 9$). The second population of samples were Namibian samples ($n = 55$) submitted to DVTD for haemoparasites testing due to suspicion of an infection. The blood samples from Namibia were spotted on Whatman® FTA cards. All results were compared with the RLB hybridization assay results.

Diagnostic sensitivity and specificity of the PCR assay were estimated in the absence of a gold standard assay, by using a two-test two-population Bayesian latent class model that allows for conditional dependence between tests (Branscum et al., 2005; Georgiadis et al., 2003). We assumed sensitivities and specificities were constant in the two populations (i.e. samples collected in South Africa and Namibia). Expert opinions were sought where no published references existed (Table 4).

The model was run in OpenBUGS, version 3.2.3 rev 1012, a programme for Bayesian analysis of complex statistical models using Markov chain Monte Carlo (MCMC) techniques (Gelfand and Smith, 1990; Lunn et al., 2009)(Appendix B). Two chains were used and initial values were generated by forward sampling from the prior distribution for each parameter. The first 10,000 iterations were discarded and the next 50,000 iterations used for posterior inferences. Model convergence was assessed by visual inspection of the trace plots.

Table 3
List of pathogens tested to determine the specificity of the TaqMan® MGB real-time PCR assay.

DNA sample	Reference
Anaplasma centrale	Frozen Anaplasmosis (Tick-Borne Gallsickness), Blood vaccine (Onderstepoort Biological Products) Reg. No.: G 1106 (Act 36/1947)
Babesia bigemina	Frozen African Redwater, Blood vaccine (Onderstepoort Biological Products) Reg. No.: G 1175 (Act 36/1947)
Babesia bovis	Frozen Asiatic Redwater, Blood vaccine (Onderstepoort Biological Products) Reg.No.: G 1106 (Act 36/1947)
Babesia caballi	Tissue culture obtained from Onderstepoort Veterinary Institute as a diagnostic sample
Babesia occultans	Tissue culture obtained from Onderstepoort Veterinary Institute as a diagnostic sample
Ehrlichia ruminantium	Heartwater - Infective Blood, Blood vaccine (Onderstepoort Biological Products) Reg. No.: G 0106 (Act 36/1947)
Theileria equi	RLB12/058
Theileria mutans	RE18/008
Theileria taurotragi	RE18/008
Theileria velifera	RE18/008
Babesia rossi	Plasmid constructed in-house
Babesia vogeli	Plasmid constructed in-house
Anaplasma platys	Plasmid constructed in-house
Anaplasma Phagocytophilum	Positive microbiome sequence analysis sample (Kolo et al., 2020)
Anaplasma sp. SA dog	Positive microbiome sequence analysis sample (Kolo et al., 2020)

Table 4
Prior values (mode and α and β-values of the beta distribution) used in a Bayesian latent class model for estimating the diagnostic sensitivity and specificity of a TaqMan® real-time PCR assay to detect *E. canis*. Pi1 - prevalence of *E. canis* in South African samples, Pi2 – prevalence of *E. canis* in Namibian samples.

	Mode	5/95th percentile	α-value	β-value	Reference
Sensitivity of RLB assay	0.85	0.70	23.90	5.04	–
Specificity of RLB assay	0.90	0.75	22.98	3.44	–
Sensitivity of PCR assay	0.90	0.20	1.94	1.10	Uniform prior
Specificity of PCR assay	0.90	0.20	1.94	1.10	Uniform prior
Pi1 (South Africa)	0.08	0.01	1.46	6.24	Prof A. Leisewitz, per. comm.
Pi2 (Namibia)	0.40	0.20	5.03	7.04	Prof B. Penzhorn, per. comm.

3. Results

3.1. TaqMan® MGB real-time PCR assay design

A total of 316 *E. canis* 16S rRNA sequences were downloaded and aligned. GenBank sequence NR_118741 was used as the reference sequence. Sequences AY394465, KF536734, EU376116, KF536738, and KF536737 were removed due to the poor quality of the sequences, as well as sequences with two nucleotide indels, which left 166 sequences. There were multiple identical sequences and only 64 unique sequences.

The nucleotide variation within the *E. canis* 16S rRNA sequences downloaded from GenBank was plotted, which showed that there was a high degree of variation at the 5′ end where a previously published assay was designed (Wen et al., 1997) and the 3′ end was relatively conserved (Appendix C).

The assay that was designed included a forward primer with redundant nucleotides, due to differences amongst the different species sequences in the *Ehrlichia/Anaplasma* genus. A species-specific probe for *E. canis* was designed, tested, and validated (Table 1). An alignment with different species in the *Ehrlichia/Anaplasma* genus shows the variation amongst the species in the *E. canis* probe region (Fig. 1).

A BLAST of the probe performed in GenBank® for homologous and heterologous sequences did not return any notable results, indicating high *in silico* specificity of the TaqMan® MGB real-time PCR assay.

3.2. Primer and probe optimization

Four different primer concentrations were tested for optimization and the lowest, efficient primer concentration was determined to be 200 nM, as it yielded a low C_T value and a steep, efficient amplification curve (Fig. 2).

The probe optimum concentration was determined by testing five different concentrations and the optimum probe concentration selected was 250 nM (Fig. 3).

3.3. Construction of a plasmid positive control

Primers (Table 2) successfully amplified a 289 bp region which included the TaqMan® MGB real-time PCR assay region of the *E. canis* 16S rRNA gene and after the fragment was sequenced 100% identity with *E. canis* sequences was obtained. The plasmid DNA was quantified as 5.8 × 10 ˆ9 plasmid/µl.

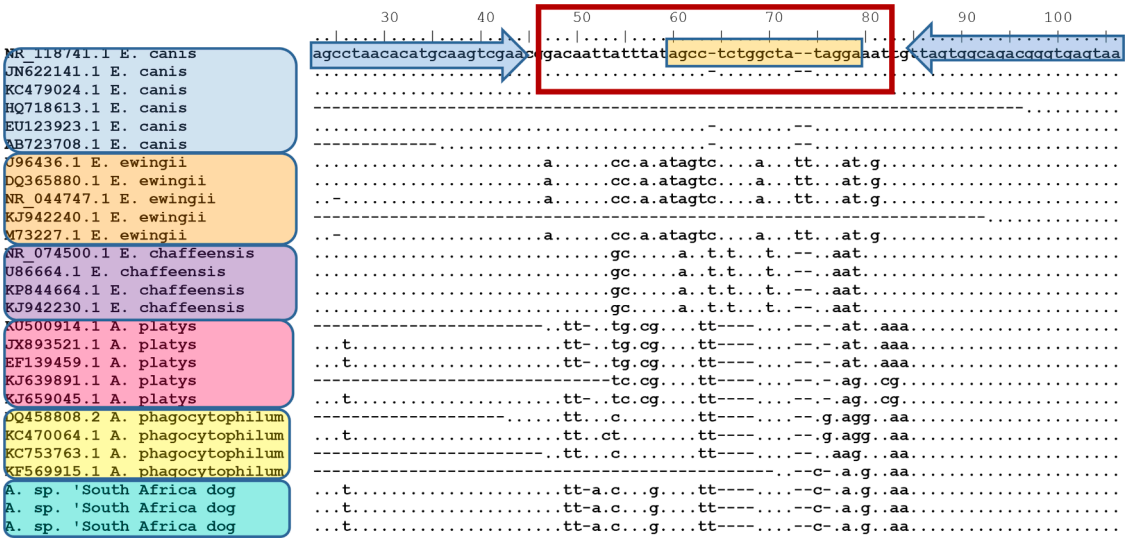

Fig. 1. Variation of aligned *Ehrlichia* spp. sequences. The primers, probe, and V1 region of the *Ehrlichia* 16S rRNA of a TaqMan® MGB assay to detect *E. canis* are represented by blue arrows, a yellow rectangle, and a red rectangular line, respectively. Sequences are identified by the accession number. The dots represent identical nucleotides to the reference sequence and the dashed represent gaps. NR_118741 was used as a reference sequence.

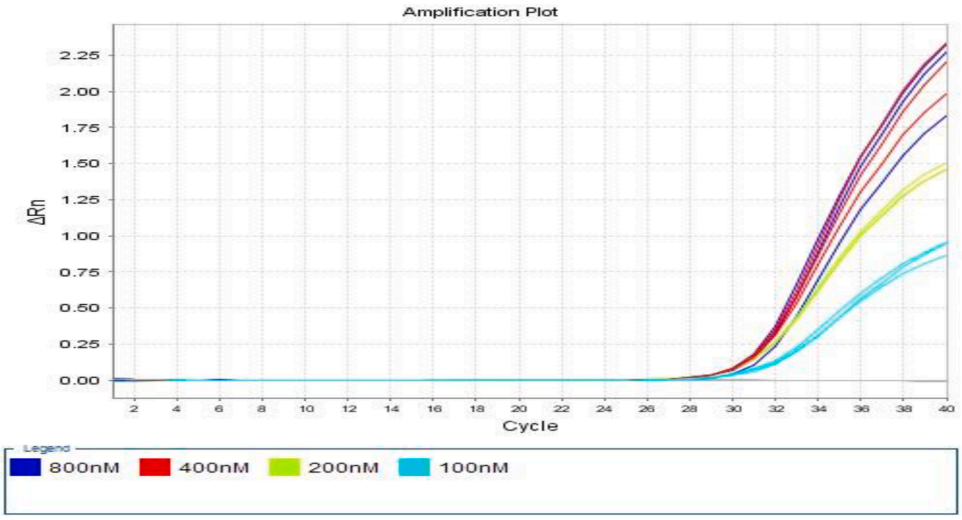

Fig. 2. Primer concentration optimization curve of a TaqMan® MGB assay to detect E. canis.

3.4. Nucleic acid purification

The digestion workflow proved to be more appropriate for the purification of nucleic acid from Whatman® FTA cards, as the average difference between the two protocols was approximately three C_T's lower using the digestion workflow, which equated to a 7.2-fold increase in sensitivity (using an efficiency of 93% for the assay) (Table 5).

3.5. Laboratory validation

3.5.1. Assay linear range and efficiency

A standard curve was generated from canine blood spiked with *E. canis* plasmid to analyse the efficiency of the assay. The assay was linear between $10^{1.43}$ to $10^{7.43}$ plasmid copies/µl blood. The efficiency of the assay was 93%, while the correlation coefficient (R^2) was used as a measure of how well the data fit the model and reflects the linearity of the standard curve, and was calculated as 0.9923 (Fig. 4).

3.5.2. Analytical sensitivity

A two-fold dilution series of blood spiked with plasmid control DNA was made to cover the nonlinear range of the assay at the LOD. The 95% LOD was 33.38 *E. canis* plasmid copies/µl of blood with a 95% confidence interval of 22.87 – 58.04 (Fig. 5).

To categorise positive and negative samples a cut-off C_T value of 37 was selected based on an approximate 50% LOD ($C_T = 37.2$) and the C_T value rounded down. Above this value, samples were classified as negative, and below this value as positive. This value equated to 7.18 *E. canis* plasmid copies /µl or a 55% LOD.

3.5.3. Analytical specificity

None of the pathogens tested (Table 3) cross-reacted with the TaqMan® MGB real-time PCR assay and only the *E. canis* positive control amplified.

3.5.4. Repeatability

Intra- and inter-run standard deviation (SD) and coefficient of variation (CV) were used to evaluate the repeatability of the assay. The inter-run standard deviation (SD) ranged between 0.33 - 1.29 and the intra-run SD 0.04- 1.14. The coefficient of variation (CV) which indicates the variation between replicates and different runs ranged between 0.12 and

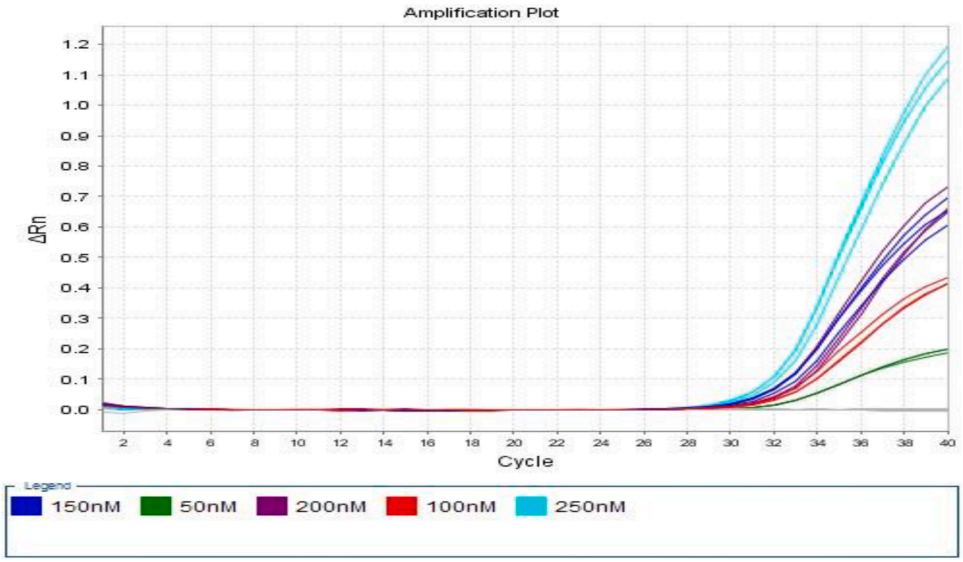

Fig. 3. Probe concentration optimization curve of a TaqMan® MGB assay to detect E. canis.

Table 5
Comparison of real-time PCR results between MagMAX™ CORE Nucleic Acid Purification Kit complex and digestion extraction methods. C_T – cycle threshold.

Sample ID	Complex workflow (C_T-value)	Digestion workflow (C_T-value)	C_T-difference
Sample 1	32.52	30.79	1.73
Sample 2	31.41	30.75	0.66
Sample 3	36.10	29.58	6.52
Sample 4	34.93	31.82	3.11
Sample 5	32.52	31.75	0.77
Sample 6	32.41	30.76	1.65
Sample 7	34.95	28.75	6.20
Sample 8	Undetermined	29.68	10.32
Sample 9	31.39	27.98	3.41

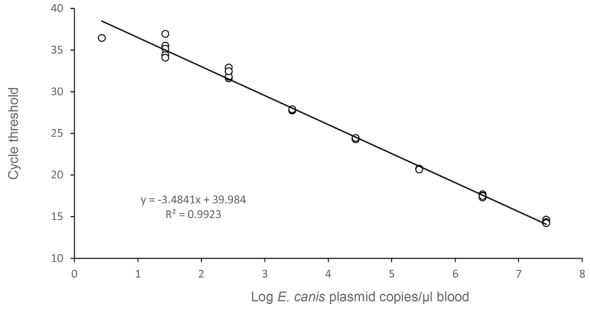

Fig. 4. Standard curve displaying the linearity of the TaqMan® MGB real-time PCR assay from 101.43 to 107.43 plasmid copies/µl blood. The efficiency of the assay was 93%.

2.9 (Table 6).

3.6. Diagnostic validation

The results for samples tested with the *E. canis* TaqMan® MGB real-time PCR assay and the RLB hybridization assay were in agreement (Table 7), with the exception of two samples. Sample RE16/087 was positive (C_T of 32.97) with the qPCR assay and negative with the RLB hybridization assay. Sample RE16/103 was positive (C_T of 35.92) with the qPCR assay and negative for *E. canis* on the RLB hybridization assay, but positive with the *B. vogeli, Theileria/Babesia* group-specific, *Babesia*1

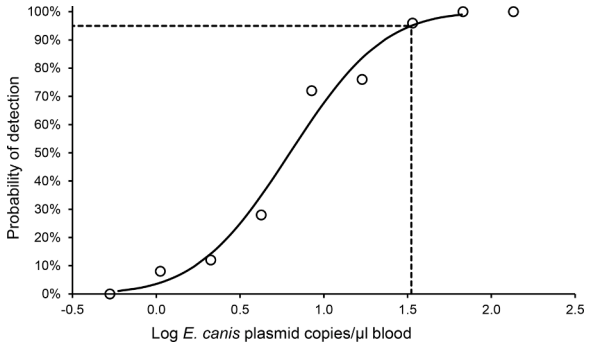

Fig. 5. The 95% limit of detection (dotted line) of a TaqMan® MGB assay to detect *E. canis* 16S rRNA gene.

Table 6
Variation of a TaqMan® MGB assay to detect *E. canis* plasmid of five replicates run in five runs. SD = standard deviation and CV = coefficient of variation.

Plasmid copies/µl blood	Inter-run SD	Intra-run SD	Total C_T Mean	Total SD	CV %
135.41	0.57	0.72	34.76	0.84	2.42
67.71	0.33	0.84	35.58	0.85	2.38
33.85	0.45	0.85	36.17	0.88	2.44
16.93	0.59	1.14	37.90	1.10	2.89
8.46	1.05	0.67	38.10	1.06	2.79
4.23	1.29	0.48	38.30	1.09	2.86
2.12	–	0.04	38.90	0.05	0.12
1.06	–	–	38.80	0.42	1.08

Table 7
Agreement/disagreement of the results of testing dog samples from South Africa and Namibia with the RLB hybridization assay and TaqMan® MGB assay specific for *E. canis* (PCR).

	Namibia samples		S. African samples	
	PCR+	PCR-	PCR+	PCR-
RLB+	9	0	31	0
RLB-	0	57	2	22

group-specific probes. Cohen's Kappa analysis was performed using the "vcd" package (Meyer et al., 2013) in R studio (RStudio Team, 2022) and the value obtained was: 0.96, p-value <0.0005 which is an almost perfect agreement. The overall agreement between the two tests was 98.3%.

Using a Bayesian latent class model (Appendix B), the sensitivity of the TaqMan® MGB real-time PCR assay was 90.17% (95% probability interval: 78.69% - 97.30%), which was slightly higher than the sensitivity of the RLB hybridization assay 86.62% (95% probability interval 76.2% - 93.73%). The specificity of the qPCR and RLB hybridization assay were similar (92.54%, 95% probability interval of 84.29% - 97.98% for the qPCR assay and 92.49%, 95% probability interval of 84.44% −97.68% for the RLB assay).

4. Discussion

Molecular detection has advantages such as early diagnosis of the disease before antibodies are produced and higher sensitivity. PCR indicates presence of the pathogen, instead of antibodies to the pathogen, which shows previous exposure. The cost of molecular assays is higher than the conventional approaches, another drawback includes false-positive detections from background DNA contamination (Yang and Rothman, 2004). New species detection and identification of closely related *Ehrlichia* spp. using group-specific primers and sequencing can be achieved by using real-time PCR (Iqbal et al., 1994; Nazari et al., 2013). Real-time PCR has caused broader recognition of PCR because it is more rapid, sensitive, and reproducible, while the risk of carryover contamination is lessened (Mackay, 2004).

We developed and validated a species-specific real-time PCR assay to detect *Ehrlichia canis* DNA in dogs, by designing an *E. canis* TaqMan® MGB real-time PCR assay targeting the 16S rRNA gene with genus-specific primers and a species-specific probe. The assay primers were designed to include all nucleotide variation within the target region and comprised a redundancy in the forward primer to allow for the detection of different species in the genus. Species-specific probes for *E. chaffeensis, E. ewingii, A. platys, A. phagocytophilum*, and *Anaplasma* sp. South Africa dog in addition to *E. canis* were designed, but not validated. The 16S rRNA gene (hypervariable region 1) otherwise known as the 30S small subunit was chosen as the target gene for the development of the TaqMan® MGB real-time PCR assay. This gene is highly conserved and is frequently used for classification of bacteria and development of group- or species-specific probes (Bottger, 1989; Clarridge, 2004). Diverse regions for species-specific probe design are limited in the 16S rRNA gene, therefore, designing probes to distinguish species in a particular genus can be challenging. However, TaqMan® MGB probes allow shorter probe design, which are useful for targeting shorter species-conserved regions. TaqMan® MGB probes also have increased assay sensitivity due to lower background signal since the 3′-end has a non-fluorescent quencher-MGB attached to it (Kutyavin et al., 2000). The developed real-time PCR assay probe is situated in the V1 which was found to be the most diverse amongst *Ehrlichia* 16S rRNA sequences (Su et al., 2021).

Primers and probe designed for this study successfully amplified and detected *E. canis* DNA in samples, producing upright sigmoidal amplification curves with low C_T-values. Optimization of the primers and probe concentration (200 nM and 250 nM, respectively) were similar to what was obtained in a multiplex real-time PCR assay for canine haemoparasites *Babesia rossi* and *B. vogeli* (Troskie et al., 2019).

We extracted DNA for the first time from Whatman® FTA cards using the digestive work and complex methods. The favourable outcome of the extraction was tested by running the known positive samples on a real-time PCR assay. The complex workflow proved to be more appropriate for Whatman® FTA extraction by yielding lower C_T values compared to those of the complex workflow.

In order to validate this assay, we simulated *E. canis* positive samples by spiking blood from an *E. canis* negative donor dog with different concentrations of plasmid DNA containing the target gene and determined the efficiency of the assay as the slope of the semi-log regression line plot of C_T-value plotted against $\log_{10}$ of input nucleic acid. There are numerous factors which can affect the efficiency of PCR amplification. These include magnesium and salt concentrations, reaction conditions, PCR target size and composition, primer sequences, and sample purity (Heid et al., 1996; Nolan and Bustin, 2013). The efficiency of the assay was 93%, which was higher than 88% obtained in a multiplex assay for *E. canis* and *B. vogeli* (Peleg et al., 2010). The high efficiency of this assay suggests that there was no primer-primer or probe-primer competition forming non-specific products. The 93% efficiency was within the acceptable range of 90% - 110% (Rogers-Broadway and Karteris, 2015).

The TaqMan® MGB real-time PCR assay was shown to be highly sensitive, by detecting 7.18 *E. canis* plasmid copies/μl (55% LOD). The assay was also more sensitive than other molecular assays that target different genes, such as a triplex qPCR assay to detect *E. chaffeensis, E. ewingii*, and *E. canis* targeting the *dsb* gene and species-specific TaqMan® probes, which had an analytical sensitivity of 50 copies per reaction (Doyle et al., 2005). The multiplex real-time qPCR published by Peleg et al. (2010) could detect up to approximately 10 copies/μl, which makes the TaqMan® MGB real-time PCR assay a sensitive tool to detect positive samples with low parasitaemia, as well as a specific tool, as no cross-reactivity was observed when tested against other haemoparasites. Previous investigations revealed *E. canis* infection parasitemia can be as low as 0.2% which is equivalent to 1 out of 500 infected circulating white blood cells (Parmar et al., 2013).

The 95% limit of detection (LOD) was 33.38 *E. canis* plasmid copies/μl. A C_T-value of 37 was determined as the cut-off to categorise positive and negative samples from the 55% LOD using the regression equation for efficiency. This value is similar to the C_T–value of 38 that was selected by Modarelli et al. (2019) as acceptable to detect potentially weak positive samples. Bhoora et al. (2018) reported a corresponding cut-off range of 35 – 37 for their multiplex assay.

A total of 121 field samples preserved on Whatman® FTA cards and blood samples were tested with both the RLB hybridization assay and the TaqMan® MGB real-time PCR assay (Appendix A). There were two disagreements between the assays, where negative results were obtained by the RLB assay and positive results by the PCR assay.

Analysis by Bayesian latent class modelling showed that the TaqMan® MGB real-time PCR assay is more sensitive and as specific as the RLB hybridization assay.

Conclusion

The *E. canis* 16S rRNA TaqMan® real-time PCR assay is a rapid, reliable, sensitive, and specific method for detecting *E. canis* DNA. The assay can correctly and rapidly diagnose CME in dogs. This will allow for treatment to be administered in the early stages of the disease, speeding up the recovery time in affected dogs. Furthermore, it will limit shotgun treatment procedures and prevent the inappropriate use of antibiotics, minimising the potential for resistant strains to emerge.

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CRediT authorship contribution statement

Nokuzola F. Nkosi: Methodology, Validation, Investigation, Data curation, Writing – original draft, Visualization, Project administration. **Marinda C. Oosthuizen:** Validation, Writing – review & editing, Supervision. **Melvyn Quan:** Conceptualization, Software, Validation,

Formal analysis, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

No potential conflict of interest was reported by the authors.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ttbdis.2022.102055.

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