Development and validation of a real-time PCR assay, and phylogenetic analysis of *Anaplasma platys*

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Abstract

Anaplasmosis is a tick-borne disease caused by species of the genus *Anaplasma.* In dogs anaplasmosis is caused by *Anaplasma phagocytophilum* and *Anaplasma platys*. These bacteria are in the family *Anaplasmataceae* in the order *Rickettsiales*. *Anaplasma platys* is a Gram-

negative bacterium that is of public health and veterinary importance. This pathogen exclusively infects platelets and causes infectious cyclic thrombocytopenia in dogs. Infection occurs through the bite of an infected ixodid tick, *Rhipicephalus sanguineus,* which is the principal vector and is also known to transmit *Ehrlichia* canis, another bacteria of veterinary importance. Our group recently reported on the developed group-specific *Ehrlichia*/*Anaplasma* primers and the *A. platys*-specific TaqMan® Minor Groove Binder probe for multiplexing purposes. This study validated the *A. platys* TaqMan® PCR assay targeting the 16S rRNA gene. Furthermore, phylogenetic analysis was used to characterize *A. platys*. The assay efficiency was 94.9%, and the 95% limit of detection (LOD) was 5.08 *A. platys* plasmid copies/µl blood with a 95% confidence interval of 3.1 - 10.2. The assay did not cross-react when tested against other haemoparasites. The phylogenetic characterization of the Mnisi community samples revealed that the *A. platys* sequences from this area grouped with other *A. platys* sequences from South Africa and other countries, including India, Zambia, Taiwan, Thailand, and Croatia. The developed TaqMan® qPCR assay will be a valuable tool for the early diagnosis of *A. platys* by preventing inappropriate use of antibiotics and alleviating potential emerging antimicrobial resistance. Additionally, early detection and administration of the correct antibiotics speed recovery time.

Keywords: Anaplasmosis, haemoparasite, dog, TaqMan®

1. Introduction

Anaplasma platys (formerly *Ehrlichia platys*) is a Gram-negative obligate intracellular alphaproteobacterium (family *Anaplasmataceae*, order *Rickettsiales*) that causes infectious canine cyclic thrombocytopenia (ICCT) (Dumler et al., 2001; Harrus et al., 1997; Harvey et al., 1978; Rar et al., 2021). This agent was first described by Harvey et al. (1978) as a small inclusion in the platelets of a dog in Florida (Dumler et al., 2001; Harvey et al., 1978). This

pathogen is the only intracellular pathogen known to infect platelets in the host (Dumler et al., 2001). *Anaplasma platys* infect canines, which are deemed as the natural host for the pathogen; however, it has also been reported in cats, foxes, red deer, goat, sika deer, cattle, Bactrian camels, and humans (Arraga-Alvarado et al., 2014; Battilani et al., 2017; Harvey et al., 1978; Maggi et al., 2013). Clinical infections in humans have been reported (Arraga-Alvarado et al., 2014; Breitschwerdt et al., 2014; Maggi et al., 2013).

Although the brown dog tick, *Rhipicephalus sanguineus* sensu lato, was not experimentally able to transmit *A. platys* to naive dogs, it is still considered the primary vector (Simpson et al., 1991).

Naturally infected hosts generally exhibit mild infection, while more severe clinical signs such as fever, lethargy, pale mucous membranes, petechial haemorrhages, epistaxis, and lymphadenopathy may occur. *Anaplasma platys* parasitaemia occurs cyclically with thrombocytopenia over several cycles between 7–14 days in length. Thrombocytopenia continues even after inclusions are no longer present in blood platelets (Eddlestone et al., 2007; Harrus et al., 1997). When the parasitaemia is low, it is challenging to distinguish *A. platys* infection from *Ehrlichia canis*, *Babesia canis*, *Babesia vogeli,* and *Rickettsia rickettsii,* which are tick-borne pathogens that can also cause thrombocytopenia. Co-infection with other pathogens can significantly influence the severity of the disease and occurs commonly due to common vectors for multiple pathogens (Eddlestone et al., 2007; Harrus et al., 1997; Hua et al., 2000; Kordick et al., 1999).

Anaplasma platys has not been successfully cultured to date. Current diagnostic methods include microscopy, where morulae in the buffy-coat smears stained with Romanowsky-type stains such as Giemsa or Diff-Quik can be seen, serology by indirect immunofluorescence, polymerase chain reaction (PCR) and reverse line blot (RLB) hybridisation assay (Ferreira et

al., 2007; Harrus et al., 1997; Martin et al., 2005). Nevertheless, these methods have shortcomings. Microscopy is unreliable due to cyclic parasitaemia. Identification of morulae in platelets is challenging and requires experience. Platelet counts decline during infection, resulting in a reduced number of circulating microorganisms, which compounds the inaccuracy of the microscopy (Ferreira et al., 2007). Serological tests are neither sensitive nor specific enough and do not detect the presence of the pathogen but rather exposure to the pathogen (Inokuma et al., 2001). The PCR and RLB assays detect the presence of pathogen DNA and are sensitive and specific. Nested PCR was considered more sensitive than straight conventional PCR, however, it is more prone to cross-contamination. Using these PCR-based methods, *A. platys* has been detected in unusual hosts, such as cattle (Akwongo and Byaruhanga, 2024) and various African tick species (Cossu et al., 2023); however, the possible cross-reactions of *A. platys* primers with recently reported novel *Anaplasma* genotypes (Makgabo et al., 2023) cast doubt upon these findings.

The genetic markers that have been used for the detection of *A. platys* PCR include *gltA*, 16S rRNA, 23S rRNA, and *GroEL* (Beall et al., 2008; Dahmani et al., 2015; Kolo et al., 2020; Lorusso et al., 2016; Rassouli et al., 2020; Sanogo et al., 2003; Vlahakis et al., 2018; Zobba et al., 2022). Gaunt et al. (2010) developed a quantitative real-time PCR (qPCR) hybridisation probe assay to detect *A. platys* based on the *p44* polynucleotide, which encodes the surface-exposed protein P44. Other *A. platys* TaqMan assays based on the 16S rRNA gene have been published; one lacked specificity since it detected other species, such as *Anaplasma phagocytophilum*, *E. canis*, and *E. chaffeensis* (Eddlestone et al., 2007). The other assay was a multiplex assay, which takes approximately 3.5 hours to process and analyse due to the additional protocol after the PCR step (Sirigireddy and Ganta, 2005). A qPCR based on TaqMan® MGB probes enables a shorter probe design to identify shorter conserved fragments within a variable region. TaqMan® qPCR assays are rapid with lesser likelihood of cross-

contamination by eliminating the need for post-run analysis, and have higher accuracy due to the nature of the probes (Kutyavin et al., 2000).

The high genetic diversity of *Anaplasma* parasites coupled with phylogenetic similarity between novel genotypes and recognised species (Makgabo et al., 2023), necessitates the development and validation of species-, strain-specific, and sequence-variants sensitive assays to avoid the cross-reactions seen in current assays. *Anaplasma platys* was found to group with *Anaplasma sp*. Mymensigh and another *Anaplasma* sp. (Kolo et al., 2020); further investigation into the phylogenetic classification is needed to contribute to a more accurate diagnosis of this genus and appropriate treatment and improved prognosis. Sequencing of *A. platys* would facilitate gene mutation analysis and distinguish the variants.

This study aimed to develop and validate a 16S rRNA TaqMan® qPCR assay for *A. platys* and phylogenetically analyse *A. platys* based on the 16S rRNA gene, for more accurate diagnosis and to increase knowledge of the strains/variants circulating in the Mnisi community, Mpumalanga, South Africa. A conserved species-specific region for *A. platys* was identified, and primers and probes were designed, optimized, and validated for the TaqMan® MGB real-time PCR assay.

2. Material and methods

2.1. Study area and blood sample collection

Samples were collected from dogs presented at Themba Animal Clinic (TAC), Mamelodi Animal Health Clinic (MAHC), or Onderstepoort Veterinary Academic Hospital (OVAH) in Pretoria, Gauteng province, South Africa, from May 2022 to March 2023. Approximately 2 ml of blood from the cephalic vein was collected into ethylenediaminetetraacetic acid (EDTA) tubes from 154 dogs that presented with clinical signs such as the presence of non-regenerative

anaemia (pale mucous membranes, low haematocrit and lack of regeneration on a peripheral blood smear), thrombocytopenia on a peripheral blood smear, easily palpable lymph nodes, large spleen and chronic history of illness. The samples were transported to the Department of Veterinary Tropical Diseases (DVTD), University of Pretoria for processing. Additional blood samples (n = 103) were obtained from the DVTD biobank collected from dogs from the Mnisi community, Mpumalanga province, South Africa, from December 2016 to April 2018.

2.2. Ethical approval

The research and procedures were approved by the Animal Ethics Committee and the Research Ethics Committee of the Faculty of Veterinary Science, University of Pretoria, South Africa (Project no. V041–16 and REC060-21) and cleared under Section 20 of the Animal Health Act 1984 [Ref. 12/11/1/1/6 (2047 RJ)] by the Department of Agriculture, Land Reform and Rural Development, South Africa.

2.3. Assay design

Anaplasma platys 16S rRNA gene sequences were downloaded from GenBank® (www.ncbi.nlm.nih.gov/genbank) and aligned using the MAFFT online version 7 (https://mafft.cbrc.jp/alignment/server), using default settings (Katoh et al., 2017). Sequences were edited and aligned in BioEdit (Hall, 1999). The molecular data analysis program DAMBE removed identical sequences (Xia, 2000, 2018).

The primers used for the TaqMan® qPCR assay described in this study were redundant in covering species differences in the *Ehrlichia/Anaplasma* genus (Nkosi et al., 2022). An *A. platys*-specific TaqMan® MGB probe was designed, optimised, and validated. To check for *in silico* specificity of the TaqMan® MGB qPCR assay, a BLAST was performed in GenBank®.

2.4. Plasmid positive control

DNA from a diagnostic dog sample (RE18/103) submitted to DVTD that tested positive for *A. platys* on the RLB hybridisation assay, was used to construct a standard positive control. To generate PCR amplicons from the sample, a primer set (Table 1) targeting 267 bp of the 16S rRNA gene inclusive of the TaqMan® qPCR region, was designed using *A. platys* JX893521 as a reference sequence. Primer design was performed using PrimerQuest® online (Integrated DNA Technologies, Inc.) and synthesised by Integrated DNA Technologies (USA).

Table 1. Plasmid construction primers for amplification of 267 bp of the *A. platys* 16S rRNA gene region containing the developed qPCR assay region.

Primer name		**Primer sequence 5'-3'**	**Start**	**Stop**	**Length**	**Tm**	**GC %**
APlatys_Pos_F	Forward	TTTGATCCTGGCTCAGAACG	5	25	20	62	50
APlatys_Pos_R	Reverse	CAGCTATAGATCACTGCCTTGG	250	272	22	62	50

The PCR reaction contained 12.5 µl of 2× Phusion Flash PCR Master Mix (Thermo Fisher Scientific, USA), 0.2 µM of each primer, 2.5 µl template, and 9.5 µl distilled water to make a final volume of 25 µl. Amplification started with initial denaturing at 98°C for 10 sec, followed by 30 cycles at 98°C for 1 sec, annealing at 55°C for 5 sec, and extension at 72°C for 15 sec, with a final elongation at 72°C for 1 min. The PCR products were purified using the High Pure PCR Template Purification Kit (Roche, South Africa), cloned into a pJET1.2/blunt cloning vector (Thermo Fisher Scientific, USA), and used to transform high-efficiency *Escherichia coli* JM109 competent cells by following the manufacturer's instructions. Cultures were grown in ImMedia™ Amp liquid broth (Invitrogen, USA) at 37°C for 1h30 min, plated on ImMedia™

Amp Blue culture plates (Invitrogen, USA) following standard procedure, and incubated overnight at 37°C.

Colonies were screened for the correct DNA insert using colony PCR, containing Dream Taq buffer (Thermo Fisher Scientific, USA), 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′), by following the manufacturer's instructions. Selected colonies were grown overnight in ImMedia™ Amp liquid broth (Invitrogen, USA) at 37°C in a shaking incubator at 150 rpm. The culture was preserved in glycerol stocks prepared by adding and gently mixing 500 µl of the culture with 500 µl (50%) glycerol and stored at - 80°C until use.

Recombinant plasmids were purified from overnight cultures using the High Pure Plasmid Isolation Kit (Roche Diagnostics, Germany), following the manufacturer's instructions. An average of three readings of the eluted plasmid DNA using the BioTek™ PowerWave™ (Analytical and Diagnostic Products, South Africa) and the Trinean Xpose spectrophotometer (Anatech Instruments, South Africa) and the average of both machine readings were used to calculate the copy number (per µl) using the formula: 6.022×10^{23} (copy number/mol) × concentration (g/µl)/molar mass (g/mol). To confirm the *A. platys* insert in the plasmid, Sanger sequencing was performed on five µl of the eluted recombinant plasmid DNA by Inqaba Biotechnical Industries (Pty) Ltd™ (Pretoria, South Africa). The remaining plasmid DNA was stored at - 20°C.

2.5. Primer/probe design and optimisation

The set of qPCR group-specific primers and probe (Table 2), designed using Primer Express® version 3.0.1 (Applied Biosystems, USA) and parameters described by Nkosi et al. (2022), were used. All designed primers and the *A. platys*-specific TaqMan® Minor Groove Binder

(MGB) probe were tested for specificity *in silico* using the nucleotide Basic Local Alignment Search Tool (BLASTn) from the National Center for Biotechnology Information website (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The internal control Xeno™ probe, which comes with the VetMAX™-Plus qPCR Master Mix kit from ThermoFisher Scientific, USA, was labelled with VIC dye to assess the effectiveness of the DNA extraction process. The TaqMan® qPCR assay probe was labelled with 6-FAM dye. To optimise the primer concentration, different primer concentrations were tested in triplicate in a PCR reaction (100 nM, 200 nM, 400 nM, and 800 nM) while maintaining a constant probe concentration of 250 nM. To allow for multiplexing purposes, primer concentrations were limited. Curves were visually inspected for a steep slope viewed on a linear scale, and the one with a low cycle threshold (CT) was selected. Likewise, different probe concentrations (50 nM, 100 nM, 150 nM, and 250 nM) were tested in triplicate with the selected optimised primer concentration to optimise the probe concentration. The conditions used for this qPCR were as recommended by the manufacturer.

Table 2. Sequence and characteristics of the *Ehrlichia/Anaplasma* group-specific forward/reverse primers (F/R) and the *A. platys* species-specific probe (P).

Primer/probe name		**Primer sequence 5'-3'**	**Start**	**Stop**	**Length**	**Tm**	**GC %**
Aplatys_P152_17	Probe	TGTCGTAGCTTGCTATGAT	5	25	20	62	50
AnapEhrlichia_F121_142	Forward	AGCYTAACACATGCAAGTCGAA	Nkosi et al. (2022)				
AnapEhrlichia_R180_201	Reverse	TTACTCACCCGTCTGCCACTAA	Nkosi et al. (2022)				

2.6. Nucleic acid purification

Nucleic acid was purified from the collected blood samples using the simple flow method of the KingFisher™ Duo Prime Purification System and the MagMAX™ CORE Nucleic Acid Purification Kit (ThermoFisher Scientific, USA). About 200 µl whole blood containing 2 µl of VetMAX™ Xeno™ Internal Positive Control DNA was used for nucleic acid purification and eluted in 90 µl of elution buffer and stored at -20°C until use.

2.7. Real-time PCR

The TaqMan® qPCR was performed using the QuantStudio™ 5 Real-Time PCR System (Applied Biosystems, USA). Each reaction mixture consisted of a final concentration of 400 nM for each of the forward primer and reverse primer, 250 nM *A. platys* probe, 12.5 µl VetMAX™-Plus qPCR Master Mix, 2 µl target DNA, 1 µl VetMAX™ Xeno™ internal positive control and distilled water to make a reaction volume of 25 µl. Cycling conditions used are listed in Table 3.

Table 3. PCR conditions used for the plasmid control construction process.

PCR steps	Stage	Number of cycles	Temperature (°C)	Duration
Polymerase activation	1	1	95°C	10 min
Denaturing	2	40	95°C	15 sec
Annealing/Extension	2	40	55°C	45 sec

2.8. Analytical validation

To determine the efficiency and linear range of the qPCR, a complete blood count was performed on the ADVIA 2120 haematology System (Siemens Healthcare GmbH, Germany) on blood obtained from a donor dog free of haemoparasites (Appendix A). A serial dilution of the plasmid control ($10^{0} – 10^{-10}$) was prepared, and 10 µl of each concentration was used to spike 190 µl blood. Nucleic acid was purified as described above. The dilution series was tested

five times in one run. The mean qPCR C_T-values from the serial dilutions were plotted against their corresponding log10 concentrations to generate a standard curve. The PCR efficiency (%) was determined from the slope of the regression using the formula: Efficiency (%) = 100 x ($10^{-1/\text{slope}}$ -1) (Pfaffl, 2001; Vandesompele et al., 2002).

For the analytical sensitivity and variation, a two-fold dilution series was prepared by diluting the plasmid with water to determine the assay's detection limit covering 0 to 100% pathogen detected by the TaqMan® qPCR assay. Each dilution was DNA extracted five times, and each extraction was tested five times (total qPCR 25 reactions per dilution). The results of these runs were used to calculate the 95% limit of detection (LOD) using a probit analysis (SPSS Statistics v25, IBM Analytics, USA).

To assess the analytical specificity of the assay, in-house constructed plasmid controls for the detection of other haemoparasites, vaccines purchased from Onderstepoort Biological Products, *A. phagocytophilum* positive DNA from a previous study by Kolo et al. (2020) and RLB hybridisation assay positive diagnostic samples submitted to DVTD, University of Pretoria were tested using the TaqMan® qPCR assay. Samples tested for the specificity of the assay are listed in Table 4.

The C_T results from the analytical sensitivity experiment were used to determine the repeatability of the assay. The inter-run, intra-run, and total standard deviations were computed as the standard deviation (SD) of the means of all runs, the mean of all runs, and the SD of all replicates, respectively. The total coefficient of variation (CV) was calculated using the formula CV = total SD/(mean C_T-value of all replicates).

Table 4. List of pathogens tested to determine the specificity of the *A. platys* qPCR assay.

Species tested (DNA)	Source
Babesia caballi	Tissue culture from the Department of Veterinary Tropical Diseases
Theileria velifera	RLB23/304
Theileria equi	RLB23/303
Theileria mutants	RLB23/304
Babesia rossi	Plasmid constructed in-house
Babesia vogeli	Plasmid constructed in-house
E. canis	Plasmid constructed in-house
***Anaplasma* sp. SA dog**	DVTD 037/21
Anaplasma phagocytophilum	L610
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 1176 (Act 36/1947)
Anaplasma centrale	Frozen Anaplasmosis (Tick-Borne Gallsickness), Blood vaccine (Onderstepoort Biological Products) Reg. No.: G 1106 (Act 36/1947)
Ehrlichia ruminantium	Heartwater - Infective Blood, Blood vaccine (Onderstepoort Biological Products) Reg. No.: G 0106 (Act 36/1947)

2.9. Diagnostic performance

A total of 154 samples were collected from three different veterinary service clinics/hospital (TAC, n = 106; MAHC, n = 25; OVAH, n = 23) and additional biobank samples (n = 103) were tested using RLB, and the developed TaqMan® qPCR assay.

The 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 Mnisi Community and Gauteng).

Table 5. 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 *A. platys*. Pi1 - prevalence of *A. platys* in Mnisi Community samples, Pi2 – prevalence of *A. platys* in Gauteng samples

	Mode	5/95th percentile	α-value	β-value	Reference
Sensitivity of RLB assay	0.87	0.70	19.45	3.76	Nkosi et al., 2022
Specificity of RLB assay	0.92	0.80	30.92	3.60	Nkosi et al., 2022
Sensitivity of PCR assay	0.50	0.05	1	1	Uniform prior
Specificity of PCR assay	0.50	0.05	1	1	Uniform prior
Pi1 (Mnisi Community)	0.19	0.10	5.96	22.13	Kolo et al., 2020
Pi2 (Gauteng)	0.10	0.25	3.44	22.99	Estimated

The estimated prevalence of *A. platys* infection in dogs from the Mnisi community (prior mode) was set at 19 % (0.19) (Kolo et al., 2020), with a 5th percentile of the beta prior distribution of 0.10. The prior mode of samples collected from dogs in Gauteng with suspected *A. platys* infection was 0.10, with a 95th percentile of the beta prior distribution of 0.25 (Table 5). The Gauteng prevalence of 0.10 was estimated in the absence of a published reference and was assumed to be lower than the Mnisi prevalence, as Gauteng is an urban environment with high levels tick control in dogs. The prior modes for the sensitivity and specificity of the RLB assay were 0.87 and 0.92, respectively, with estimated 5th-percentile values of 0.70 and 0.80 (Nkosi et al., 2022).

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 Adrian, 1990; Lunn et al., 2009). Two chains were used, and initial values were generated by forward sampling from the prior distribution for each parameter. The first 20,000 iterations were discarded, and the subsequent 80,000 iterations were used for posterior inferences. Model convergence was assessed by visual inspection of the trace plots. The simulations were run until the Monte Carlo error for each parameter of interest was less than 5% of the sample standard deviation.

2.10. Phylogenetic analysis

The near full-length (1,500 bp) *Anaplasma*/*Ehrlichia* 16S rRNA gene from ten *A. platys*-RLB and qPCR strong positive samples from DVTD were amplified using primers fD1 (5'- AGA GTT TGA TCC TGG CTC AG -3') and rP2 (5'- ACG GCT ACC TTG TTA CGA CTT -3') described by Weisburg et al. (1991). The following conditions, initial denaturation 98°C for 10 sec, denaturation 98°C for 1 sec, annealing 55°C for 5 sec, extension 72°C for 15 sec and final extension 72°C for 1 min were used to amplify the 16S rRNA gene using the Phusion flash master mix (Thermo Fisher Scientific, USA). The PCR products were sent to Inqaba Biotechnical Industries (Pty) Ltd™ (Pretoria, South Africa) for direct Sanger sequencing. The sequencing results were analysed and trimmed using CLC Genomic Workbench 7 software (Qiagen bioinformatics). The sequences were aligned using MAFFT online (https://mafft.cbrc.jp/alignment/server) using default settings. The *A. platys* sequences were subjected to nucleotide Basic Local Alignment Search Tool (BLASTn) check for similar sequences and then used to construct a phylogenetic tree. The phylogenetic tree for the 16S rRNA gene sequences was constructed using the maximum likelihood (ML) method in MEGA

12 with 1000 bootstraps replications, and the optimal General Time Reversible Model was chosen (Kumar et al., 2024).

3. Results

3.1. Assay design

A total of 153 *A. platys* GenBank 16S rRNA sequences were downloaded, aligned, and cleaned, after removing low-quality and identical sequences in DAMBE, only 59 sequences remained for further analysis. The *Ehrlichia/Anaplasma* genera-specific TaqMan® qPCR primers, as described by Nkosi et al. (2022), and an *A. platys*-specific TaqMan® MGB probe was designed, optimised, and validated (Table 2). The *in silico* specificity check on the TaqMan® MGB qPCR assay primers and probe in BLAST performed in GenBank® showed no homologous or heterologous sequences were detected.

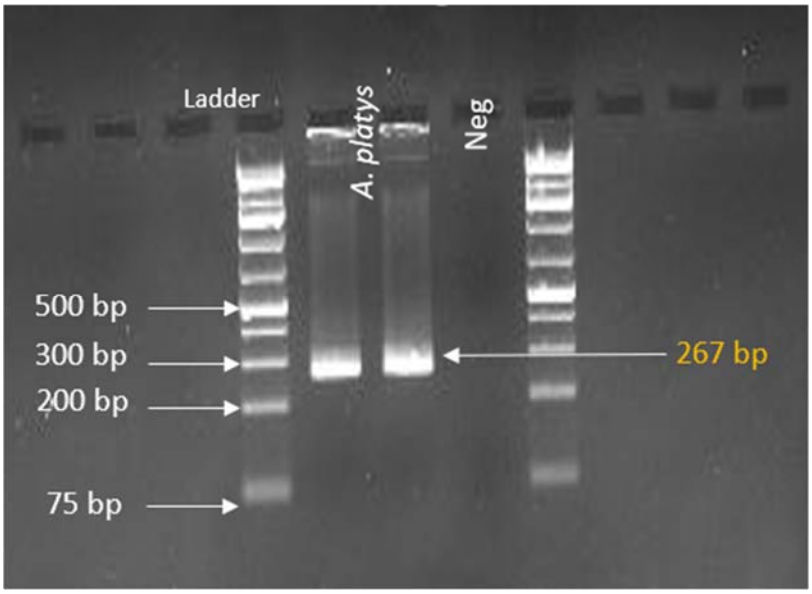

Figure 1. Visualisation of two 267 bp *A. platys* amplicons on a 2% agarose gel. The sizes of the bands were compared to a GeneRuler™ 1kb Plus DNA ladder (Thermo Scientific™, USA) in the outer wells.

3.2. *In-house plasmid control*

The 267 bp region of the *A. platys* 16S rRNA gene (Figure 1), inclusive of the developed TaqMan® MGB qPCR, was successfully amplified with the designed cloning primers (Table 1). Sequencing results showed 100% identity to *A. platys,* and the plasmid concentration was calculated at 5.2×10^9 plasmid/µl.

3.3. *Primers and probe optimisation*

The lowest primer concentration that efficiently amplified targets was 400 nM. Using 400 nM of primer concentration in the TaqMan® qPCR mix, the probe's optimal concentration was 250 nM.

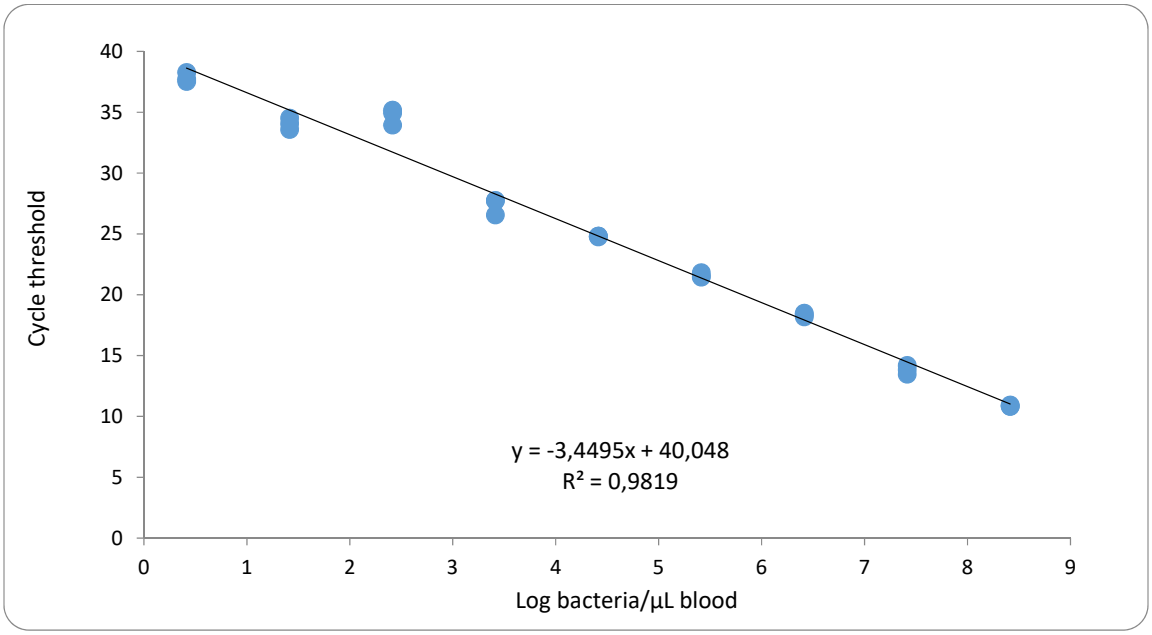

Figure 2. Standard curve displaying the linearity of the *A. platys* qPCR assay. The efficiency was 94.9%.

3.4. Analytical validation

The *A. platys* TaqMan® qPCR was linear between $10^{1.43}$ and $10^{7.43}$ plasmid copies/µl blood, with the analyses from the blood spiked with the *A. platys* plasmid. The linear regression of the assay was specified as $R^2 = 0.98$, and the efficiency of the assay was 94.9% (Figure 2).

The assay demonstrated high sensitivity, with a 95% LOD of 5.08 *A. platys* plasmid copies/µl of blood and a 95% confidence interval of 3.1 - 10.2 (Figure 3). The cut-off value was a rounded-off value of a C_T of 38, and it was selected based on the 95% LOD (C_T = 37.6); samples with a C_T-value equal to or below this were classified as positive, and all samples with values above this were regarded as negative.

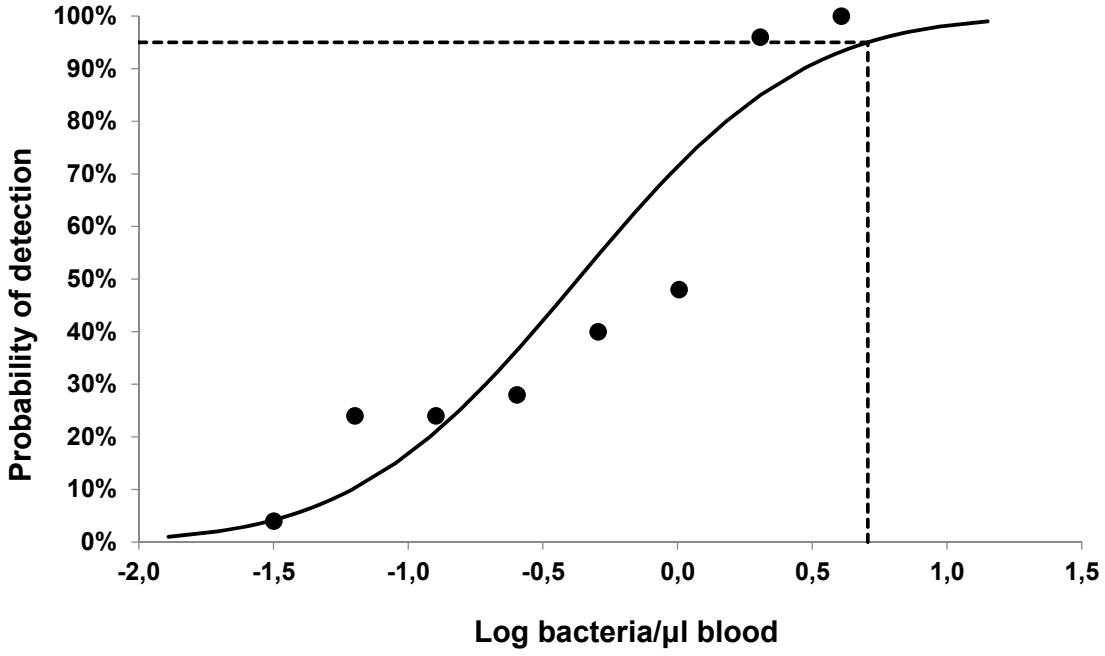

Figure 3. The 95% limit of detection (dashed line) of the TaqMan® MGB qPCR assay for detecting the *A. platys* 16S rRNA gene.

None of the pathogens listed in (Table 4) cross-reacted when tested with the developed *A. platys* TaqMan® qPCR; only the positive control amplified (*A. platys* plasmid was positive).

Table 6. The TaqMan® qPCR for A. platys plasmid detection was evaluated over five runs with five replicates each, using standard deviation (SD) and coefficient of variation (CV) for performance assessment.

Plasmid copies/µl blood	Inter-run SD	Intra-run SD	Total CT Mean	Total SD	CV %
129,70	0,27	0,04	30,44	0,32	1,04
64,85	0,38	0,07	31,44	0,39	1,23
32,42	0,30	0,08	32,56	0,36	1,11
16,21	0,23	0,17	33,47	0,39	1,15
8,11	0,31	0,16	34,45	0,49	1,42
4,05	0,41	0,47	35,58	1,03	2,89
2,03	3,35	6,98	34,97	7,32	20,92
1,01	3,99	1,08	17,57	18,67	106,26
0,51	8,91	8,77	14,58	18,24	125,04
0,25	12,54	9,51	10,50	17,19	163,67
0,13	9,98	10,72	9,19	16,69	181,63
0,06	6,12	8,11	8,88	16,14	181,76
0,03	3,37	7,54	1,51	7,54	500,00

The repeatability of the assay was assessed with the intra- and inter-run standard deviation (SD) and coefficient of variation (CV). The inter-run standard deviation (SD) ranged between 0.2 and 12.5, and the intra-run SD was 0.04 and 10.7. The coefficient of variation (CV), which indicates the variation between replicates and different runs, ranged between 1.04 and 500 (Table 6).

3.5. Diagnostic performance

The 257 field and biobank samples were tested with the *A. platys* TaqMan® qPCR and RLB assays. The results of the two tests correlated in 92.6% of the tested samples, and 7.4% were conflicting and inconsistent: 3.9% (n = 10) of samples were positive with the RLB assay and

negative with the *A. platys* TaqMan® qPCR, and 3.5% (n = 9) of the samples were positive with the *A. platys* TaqMan® qPCR and negative with the RLB test (Table 7).

Table 7. Agreement/disagreement of test results for canine samples from Gauteng and Mpumalanga provinces were analyzed using the RLB hybridization assay and TaqMan® qPCR assay specific for *A. platys*.

	Mpumalanga province		**Gauteng province**	
	qPCR +	qPCR -	qPCR +	qPCR -
RLB +	62	10	5	0
RLB -	9	22	0	149

The Cohen's Kappa (K) test showed a substantial agreement between the RLB and the developed qPCR ($k = 0.761$, $p < 0.001$).

Applying the Bayesian latent class model (Appendix B), the sensitivity of the TaqMan® MGB qPCR assay was estimated to be 86.13% (95% probability interval: 78.50% – 92.00%), which was marginally lower than the sensitivity of the RLB hybridisation assay, measured at 87.55% (95% probability interval: 81.13% – 92.50%). The specificity of the qPCR assay was higher, achieving 99.45% (95% probability interval: 97.64% – 99.96%) compared to 96.50% (95% probability interval: 93.20% – 98.68%) for the RLB hybridisation assay (Table 8).

Table 8. Estimates of the diagnostic sensitivity and specificity of the qPCR assay.

Test parameter	**Median**	**95% credible interval**
Sensitivity of the PCR assay	0.8613	0.7850 - 0.9200
Specificity of the PCR assay	0.9945	0.9764 - 0.9996
Sensitivity of the RLB assay	0.8755	0.8113 - 0.9250
Specificity of the RLB assay	0.9650	0.9320 - 0.9868
Sensitivity of the PCR assay	0.8613	0.7850 - 0.9200

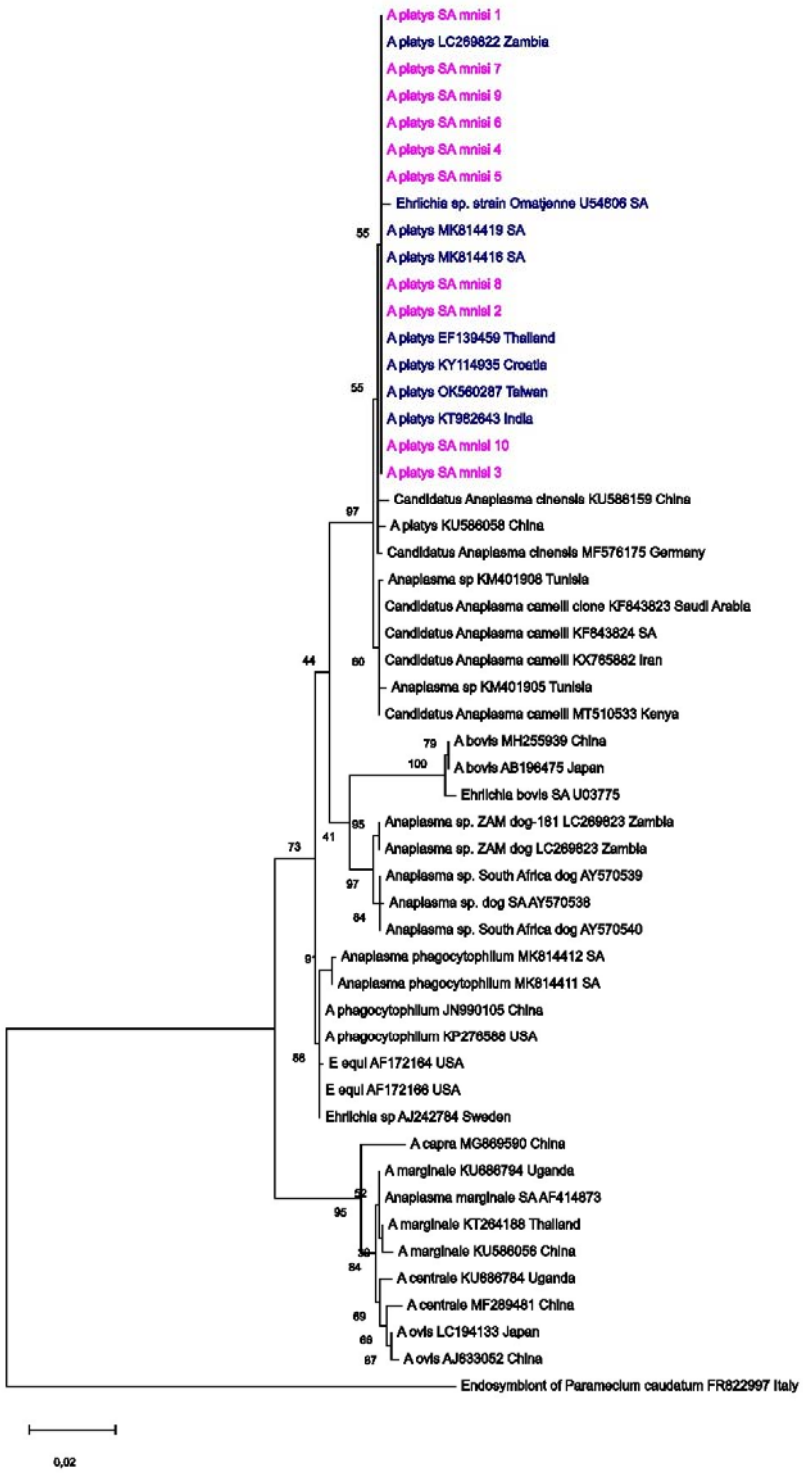

Figure 4. Maximum likelihood phylogenetic tree based on 16S rRNA sequence data showing the phylogenetic relationships between *A. platys* sequences obtained from this study (pink sequences) and

available 16S rRNA sequences from different countries, including South Africa (blue and black sequences).

3.6. Phylogenetic analysis

The relationships of the ten near-full *Anaplasma* 16S rRNA gene sequences identified in this study from the samples collected in Mpumalanga were analysed using the ML method. The results showed that all the sequenced *A. platys* samples from this study were identical as a result clustered together and were similar to other *A. platys* strains found in other countries (India, Thailand, Croatia, and Taiwan), including African countries such as Zambia (Figure 4).

4. Discussion

This study aimed to develop and validate an *A. platys* TaqMan® qPCR for the accurate and rapid diagnosis of canine anaplasmosis. This was achieved by using and optimizing the group-specific *Ehrlichia/Anaplasma* primers designed by Nkosi et al. (2022) and validating the *A. platys* species-specific TaqMan® probe. The test probe was designed in the 16S rRNA gene, which is commonly used for the classification and probe development of pathogens and the hypervariable region 1 was selected because it differs among species, allowing for species-level identification (Harrus et al., 1997; Martin et al., 2005; Pinyoowong et al., 2008). TaqMan® based test reduce processing time by eliminating the need for post-run analysis. They also enable the development of shorter probes than standard probes, which is a significant advantage for identifying conserved regions in highly variable target regions. Due to the lower background caused by the non-fluorescent quencher-MGB attached at the 3' end, better precision is also achieved with these probes and was therefore used in this study (Kutyavin et al., 2000).

This study's optimisation of primers and probe was 400nM and 250nM, respectively. The concentration obtained for the primers was slightly higher than what other studies obtained, but the probe concentration was similar (Nkosi et al., 2022; Troskie et al., 2019). Amplification and detection of *A. platys* DNA in samples was achieved, resulting in distinct sigmoidal amplification curves with low C_T-values.

Assay validation was achieved by simulating a natural infection by spiking blood from an *A. platys*-negative donor dog with different concentrations of the plasmid containing the gene of interest. We calculated the assay efficiency from the slope of the semi-log regression line and plotted the C_T-values against the logarithm of the input nucleic acid concentration. The efficiency of the assay was 94.9%, which is in the same range as other studies using TaqMan probes and is in the acceptable range of 90% to 110% (Nkosi et al., 2022; Troskie et al., 2019; Venter et al., 2024). This high efficiency indicates the absence of non-specific products formed by primer-primer or probe-primer competition. The *A. platys* TaqMan® qPCR assay demonstrated high sensitivity, detecting as few as 5.08 *A. platys* plasmid copies/µl of blood at a 95% LOD. This sensitivity exceeds that of the triplex assay for *Ehrlichia chaffeensis*, *Ehrlichia ewingii*, and *E. canis*, which detects 50 copies per reaction targeting the *dsb* gene with species-specific TaqMan® probes, as well as the 7.18 *E. canis* plasmid copies/µl (55% LOD) (Doyle et al., 2005; Nkosi et al., 2022).

Based on the efficiency regression equation, a C_T-value of 37.6, rounded to 38, was set as the cut-off value to distinguish positive from negative samples at the 95% LOD. This value is comparable to the 38 selected by Modarelli et al. (2019), 37 chosen by Nkosi et al. (2022), and the 35 – 37 cut-off range of the multiplex assay by Bhoora et al. (2009).

There were discrepancies in the results obtained from RLB and the *A. platys* TaqMan® qPCR assays, and 19 samples did not correspond for the two tests; nine were positive with the *A.*

platys TaqMan® qPCR but negative for the RLB assay, and ten were positive with the RLB assay and negative with the *A. platys* TaqMan® qPCR assay. The nine positive with the *A. platys* TaqMan® qPCR had C_T-values between 23 - 33.5. These C_T-values are below the cut-off value, and due to the sensitivity and specificity of the qPCR probes, we have classified them as true positive. It is not clear why the qPCR did not detect these samples. However, the DNA used for this test was a few years old and may have been degraded. We attempted to eliminate this analogy by retesting the samples with both tests, and we still obtained the same results. Other studies have shown that there are sequence-variants within *Anaplasma* species, thus suggesting mutations in the probe-target region of the qPCR (Makgabo et al., 2023; Wang et al., 2014). Furthermore, RLB interpretation is subject to analysis by the analyst, and the results might have appeared as *A. platys* and was analysed as such (Bhoyar et al., 2024).

The estimated sensitivity was 86.13% for the *A. platys* TaqMan® qPCR assay and 87.55% for the RLB assay, while the specificity of the developed qPCR was higher at 99.45% than 96.50% for the RLB assay. Even though the RLB's sensitivity is slightly higher, the developed qPCR proved to be a very specific tool for detecting *A. platys* DNA in canine blood. It is quicker to perform than RLB, reducing the turnaround time for diagnosis.

The phylogenetics of *A. platys* using the 16S rRNA gene with sequences generated from Mnisi community grouped with other known sequences of *A. platys* from South Africa, Ehrlichia sp. strain Omatjenne from South Africa, and *A. platys* from other countries such as India, Zambia, Taiwan, Thailand, and Croatia. The genetic similarities for India, Croatia, Thailand, and Taiwan had 100% similarities, while the Ehrlichia sp. strain Omatjenne_U54806_SA from South Africa had 99.7%. This suggests that the *A. platys* strain circulating in the Mnisi community, Bushbuckridge Municipal Area, Mpumalanga Province, South Africa, is similar to what was published in prior studies (Kolo et al., 2020). However, 16S rRNA is not ideal for

distinguishing strains and species within the genus *Anaplasma*, due to similarities of over 98.7% among unique *Anaplasma* species (Caudill and Brayton, 2022). Therefore, using the 16S rRNA gene alone cannot identify circulating strains. For such purpose, other genes such as *groEL*, *gltA*, and *Anaplasma*-specific *msp* genes within *Anaplasma* species need to be explored along with 16S rRNA for proper distinction of the various species within the *Anaplasma* genus. The use of advanced sequencing methods has led to the identification of numerous novel *Anaplasma*-like 16S rRNA gene sequences; however, their relationship to known pathogens and the possibility of testing errors remain unclear. Since these genes are highly similar, often over 98% identical, misclassification is a risk, especially when using the common 97% identity score. This highlights the need for caution when identifying species and designing detection tests (Caudill and Brayton, 2022).

5. Conclusion

The *A. platys* TaqMan® qPCR developed in this study is a rapid, sensitive, and specific method for detecting *A. platys* DNA. Although some RLB-positive samples were not positive when tested with the qPCR assay, the qPCR assay is faster to perform than the RLB. Since it has been proven that there are significant similarities within the genus *Anaplasma*, similarly high variations among the same species, investigation of mutations that might be occurring within the probe target region of the designed qPCR must be conducted in order to possibly improve the current discrepancies. It is likewise recommended to identify genes other than the 16S rRNA gene to distinguish *Anaplasma* species.

Declaration of competing interest

No potential conflict of interest was reported by the authors.

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Author contribution

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