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Development of a multiplex polymerase chain reaction assay for the detection of Piroplasma (*Babesia* spp. and *Theileria* spp.), *Anaplasma* spp., and *Trypanosoma evansi* in cattle

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ABSTRACT

Background: Blood parasites cause serious drawbacks in the livestock industry. The detection methods rely on microscopic examination and polymerase chain reaction (PCR), which requires an expert person, labor, time, and cost.
Aim: In this study, a multiplex PCR has been developed for hemoparasites consisting of Piroplasma (*Babesia* spp. and *Theileria* spp.), *Anaplasma* spp., and *Trypanosoma evansi* detections.
Methods: Blood parasite infections in cattle were investigated using three different methods: microscopic examination, single PCR, and multiplex PCR. The *Anaplasma* 16s rRNA gene, *Babesia* 18s rRNA gene, *Theileria major piroplasm surface protein* gene, and *Trypanosome ITS1* gene have been used to detect *Anaplasma* spp., *Babesia* spp., *Theileria* spp., and *T. evansi*, respectively, using molecular methods. The sensitivity and specificity of multiplex PCR were evaluated. Multiplex PCR results were compared with microscopic examination and single PCR.
Results: Multiplex PCR assay revealed a limit of detection of 0.01–10 pg of parasite DNA. According to the evaluation of 60 bovine blood samples, blood smear, single PCR, and multiplex PCR revealed 50.0%, 18.3%, and 26.7% of single infections, and 40.0%, 81.7%, and 50.0% of co-infections, respectively. The comparative analysis between multiplex PCR with microscopic examination and single PCR revealed that triple infection, *Babesia* spp., *Theileria* spp., and *T. evansi*, showed 50.0% sensitivity, 100% specificity, and positive predictive value, 98% negative predictive value, and substantial agreement indicated by a Cohen's Kappa value of 0.659.
Conclusion: The multiplex PCR assay developed in this study may be helpful for improved hemoparasite prevention and control when combined with farmer education, proper hygiene practices, and effective environmental management.
Keywords: Hemoparasites, Multiplex PCR, Piroplasma, *Trypanosoma evansi.*

Introduction

Ticks are hematophagous ectoparasites of livestock in tropical and subtropical areas (Eskezia, 2016). Ticks transmit various pathogens, such as viruses, bacteria, and protozoa (Baneth, 2014). The cattle tick, *Rhipicephalus microplus*, is the most important tick affecting more than 80% of the cattle population worldwide and causes severe economic losses due to both direct (tick bites) and indirect (pathogen transmission) effects (Benavides and Romero, 2001; Hurtado and Giraldo-Ríos, 2019). *Anaplasma*, *Babesia, and Theileria* species are bovine tick-borne pathogens that cause tick fever (*Anaplasma* spp. and *Babesia* spp.) and piroplasmosis (*Babesia* spp. and *Theileria* spp.). These diseases show mild to severe symptoms, such as fever, hemolytic anemia, anorexia, abortion, and death, and in some cases lead to meat and milk production decrements, which impact the livestock industry worldwide (Suarez and Noh, 2011; Zhou *et al.*, 2016; Abdela and Bekele, 2016). *Trypanosoma evansi* is one of the blood parasites that cause surra and has a major impact on animal health in Southeast Asia

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(Suwan *et al.*, 2023). Co-infections of blood parasites are common, causing virulence, infectivity, and transmissibility of pathogens, leading to morbidity and mortality in animals (Gomez-Chamorro *et al.*, 2021).
Blood parasite diagnosis relies on the presence of intra/inter erythrocytic bodies under microscopic examination of blood smears, but requires bare observation under subclinical or low parasitemia conditions, and requires an experienced and skillful person. In addition, it was difficult to distinguish when co-infected blood parasites occurred (Ananyutthawongse *et al.*, 1999; Peng *et al.*, 2020; Kumar *et al.*, 2021). The serological assays also showed drawbacks of cross-reactivity, lack of sensitivity, and discrimination between present and previous infections (Bilgiç *et al.*, 2013; Kundave *et al.*, 2018). The molecular method of polymerase chain reaction (PCR) has shown promising sensitivity and specificity in detecting the presence and/or low parasitemia (Parodi *et al.*, 2021). However, when diagnosing large or coinfected blood parasite samples, individual PCR is expensive and time-consuming (Bilgiç *et al.*, 2013; Kumar *et al.*, 2021). Multiplex PCR has been developed for simultaneous detection of multiple pathogens in a single reaction without additional reagents or DNA template requirements (Kundave *et al.*, 2018; Hao *et al.*, 2019; Peng *et al.*, 2020) and has been used to detect many bovine hemoparasites (Ananyutthawongse *et al.*, 1999; Bilgiç *et al.*, 2013; Kundave *et al.*, 2018; Peng *et al.*, 2020; Kumar *et al.*, 2021; Parodi *et al.*, 2021). The challenge of multiplex PCR development is primer design, which requires specificity and sensitivity when the number of pathogen detections increases (Peng *et al.*, 2020).
This study aimed to develop a multiplex PCR for the detection of hemoparasites consisting of Piroplasma (*Babesia* spp. and *Theileria* spp.), *Anaplasma* spp., and *T. evansi*. Multiplex PCR reactions and conditions were optimized to detect these blood parasites. Sensitivity and specificity were evaluated. Statistical methods were used to indicate the agreement between the results of multiplex PCR and microscopic examination and single PCR.

Materials and Methods

Sample preparation

A licensed veterinarian collected 60 blood samples from the jugular vein of dairy cattle in June 2024 in Sakonnakhon province, Thailand. Blood samples (3 ml) were stored in sterile ethylene-diamine-tetraacetic acid tubes and transported within 24 hours to the Faculty of Veterinary Technology, Kasetsart University, Bangkok, Thailand. All blood samples were subjected to microscopic and molecular examinations. For microscopic examination, a Giemsa-stained thin blood smear was used to detect blood parasites under a light microscope. For molecular examination, 250 µl of blood sample was used to extract genomic DNA (gDNA) using the E.Z.N.A. Blood DNA Mini Kit (Omega Bio-tek, Georgia) according to the manufacturer's instructions. The gDNA concentration was measured using a NanoDrop Lite Spectrophotometer (Thermo Fisher Scientific Inc., USA) and kept at −20°C until use. Light microscopic examination was performed to detect hemoparasites, including *Anaplasma* sp., *Babesia* sp., *Theileria* sp., and *T. evansi*. The gDNA of positive blood samples was prepared as described above and used as a template for positive controls.

Primer design

The primers of *Theileria* spp. and *Trypanosoma* spp. were designed based on specific genes, including *major piroplasm surface protein* (MPSP) (Rakwong *et al.*, 2022) and *internal transcribed regions* (*ITS)1* (Suwan *et al.*, 2023), respectively. Primers for *Anaplasma* spp. and *Babesia* spp. were designed from *16S* and *18S ribosomal RNA*, respectively (Table 1).

Cloning of the positive controls

PCR was performed using an individual primer for each blood parasite. The PCR reaction comprised of 1× of ExcelTaq™, 5X PCR Master Mix (SMOBIO, Taiwan), 1 µM of each forward and reverse primer, and 100 ng of positive blood sample gDNA. The PCR conditions were 95°C for 5 minutes of pre-denaturation followed by 35 cycles of 95°C for 3 minutes, 50°C for 1 minute of annealing, 72°C for 1 minute of extension, and 72°C for 10 minutes of post-extension. The target genes of each blood parasite were

Table 1. List of primers used in multiplex PCR.

Pathogens	Primers	Sequences (5′-3′)	Product sizes (bp)
Anaplasma spp.	16s-F	TGA TCT ATA GCT GGT CTG AGA GG	720
	16s-R	CGC CCT TCT GTT AAG AAG GAT CTA	
Theileria spp.	MPSP-F	AAG AAG AAG ACT GAC AAG GAT TGG	436
	MPSP-R	GGA CTA CAA GCC TCT TGT CGA	
Trypanosoma evansi	ITS1-F	TAG GTG AAC CTG CAG CTG G	292
	ITS1-R	CGT ATG GAA TGC GTA TCT CTC T	
Babesia spp.	18s-F	AAA GCA TTT GCC AAG GAC GCT TC	202
	18s-R	TTG TCT GGT TAA TTC CGT TAA CGA AC	

cloned into pGEM®-T Easy Vector (Promega, USA) and transformed into *Escherichia coli* DH5α. Positive plasmids were extracted using the GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific Baltics UAB, Vilnius, Lithuania). Target genes were confirmed by sequencing (U2bio, Thailand).

Single and multiplex PCR optimizations

A single PCR was performed using an individual primer for each blood parasite. The PCR reaction comprised 1× of ExcelTaq™, 5X PCR Master Mix (SMOBIO, Taiwan), 1 µM of each specific forward and reverse primer, and 100 ng of positive plasmid. The PCR conditions were 95°C pre-denaturation for 5 minutes, followed by 35 cycles of 95°C denaturation for 3 minutes, 50°C annealing for 1 minute, 72°C extension for 1 minute, and 72°C post-extension for 10 minutes. The PCR product was verified using 1.5% agarose gel electrophoresis.

The multiplex PCR reaction comprised 1× of ExcelTaq™, 5X PCR Master Mix (SMOBIO, Taiwan), 0.5–2 µM of each forward and reverse primer, and 100 ng of each positive control. The PCR conditions were 95°C pre-denaturation for 5 minutes, followed by 35 cycles of 95°C denaturation for 3 minutes, 55°C annealing for 1 minute, 72°C extension for 1 minute of extension, and 72°C post-extension for 10 minutes. The PCR product was verified using 1.5% agarose gel electrophoresis.

Validation of the multiplex PCR

Positive controls were serially diluted 10-fold and used as a template. The sensitivity and specificity of multiplex PCR were evaluated. For specificity, 1 ng of each individual positive control was used as a template. For sensitivity, 0.00001–10 ng of the individual positive control was used as the template. The PCR reaction comprised of 1× of ExcelTaq™, 5X PCR Master Mix (SMOBIO, Taiwan), 0.5 µM of *Theileria* MPSP and *Babesia* 18s RNA, 1 µM of *Anaplasma* 16s RNA, and 2 µM of *Trypanosome* ITS forward and reverse primers, and template. The PCR conditions were 95°C pre-denaturation for 3 minutes, followed by 35 cycles of 95°C denaturation for 1 minute, 55°C annealing for 1 minute, 72°C extension for 1 minute, and 72°C post-extension for 10 minutes. The PCR product was verified using 1.5% agarose gel electrophoresis.

Field sample detection

gDNA from field blood samples was used as a template. The multiplex PCR reaction comprised 1× of ExcelTaq™, 5× PCR Master Mix (SMOBIO, Taiwan), 0.5 µM of *Theileria* MPSP and *Babesia* 18s RNA, 1 µM of *Anaplasma* 16s RNA, and 2 µM of *Trypanosome* ITS forward and reverse primers, and 1 ng of gDNA. The PCR conditions were 95°C pre-denaturation for 5 minutes, followed by 35 cycles of 95°C denaturation for 3 minutes, 55°C annealing for 1 minute, 72°C extension for 1 minute, and 72°C post-extension for 10 minutes. The PCR product was verified using 1.5% agarose gel electrophoresis.

The positive PCR products were randomly selected, and their DNA was extracted from agarose gel using the PureDireX PCR Clean-Up & Gel Extraction Kit (BIO-HELIX, Taiwan) according to the manufacturer's instructions. The extracted DNA samples were sent for sequencing (U2bio (Thailand)). The sequencing results were searched against the National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/) using the Basic Local Alignment Search Tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) (Altschul *et al.*, 1990).

Statistical analysis

All statistical analyses were conducted using R version 4.5.1 (R Core Team, 2025). The results of multiplex PCR were compared with those of microscopic examination and single PCR in terms of sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) using the "epiR" package in R. The degree of agreement was evaluated using Cohen's Kappa coefficient via the "fmsb" package in R, and the strength of agreement was interpreted according to the guidelines of Landis and Koch (1977).

Ethical approval

This study was approved by the Ethics Committee of Kasetsart University, Bangkok, Thailand (Approval no. R-ACVTN-65-002).

Results

Construction of positive controls and optimization of single and multiplex PCR

Plasmids harboring target genes of blood parasites were constructed, sequenced, and used as positive controls. Single and multiplex PCRs were performed (Fig. 1). The single PCR showed 720, 202, 436, and 292 bp PCR products corresponding to the *Anaplasma* 16s rRNA gene, *Babesia* 18s rRNA gene, *Theileria* MPSP gene, and *Trypanosome* ITS1 gene, respectively. Multiplex PCR showed the PCR products when the template concentrations in the range of 0.002–200 ng/µl of each positive control were used.

Specificity and sensitivity of multiplex PCR

Multiplex PCR showed specific bands corresponding to each blood parasite sample (Fig. 2A). The sensitivity showed a limit of detection of each blood parasite, which was 0.00001 ng/µl of *Anaplasma* 16s rRNA and *Babesia* 18s rRNA, 0.01 ng/µl of *Theileria* MPSP, and 0.0001 ng/µl of *Trypanosome* ITS1, which corresponded to 0.01 pg, 0.1 pg, and 10 pg of DNA for *Anaplasma* spp., *Babesia* spp., *Theileria* spp., and *T. evansi*, respectively (Fig. 2B–E).

Field sample detection

Blood parasites, including *Anaplasma* spp., *Babesia* spp., *Theileria* spp., and *T. evansi*., were detected in 60 bovine blood samples by microscopic examination, single PCR, and multiplex PCR (Table 2). According to light microscopy, 17 out of 60 (28.33%) and 13 out of 60 (21.66%) showed single infections of *Anaplasma* spp. and *Theileria* spp., respectively. Twenty – three samples

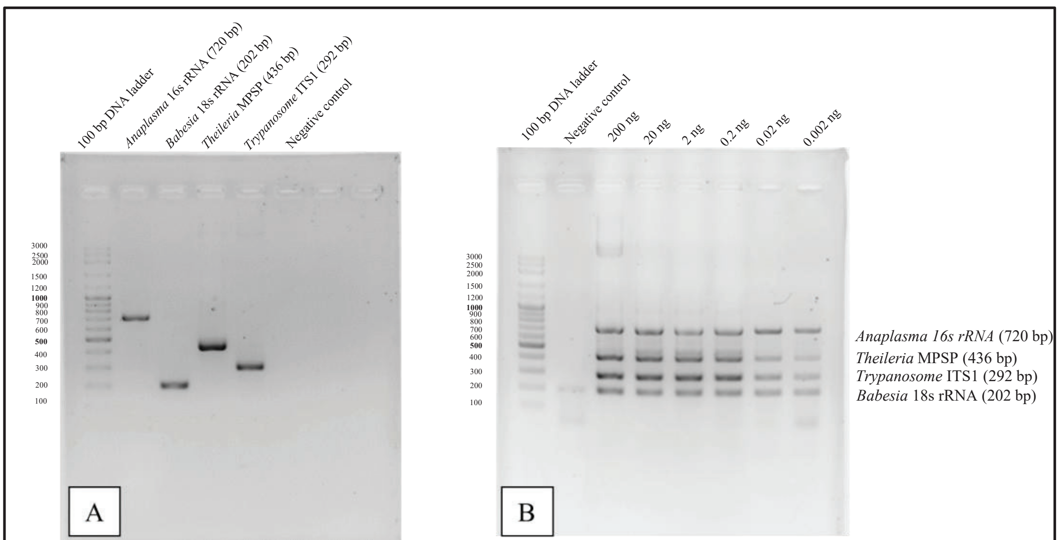

Fig. 1. Single (A) and multiplex (B) PCR. The PCR products of *the Anaplasma* 16s rRNA gene, *Babesia* 18s rRNA gene, *Theileria* MPSP gene, and *Trypanosome* ITS1 gene were 720, 202, 436, and 292 bp, respectively.

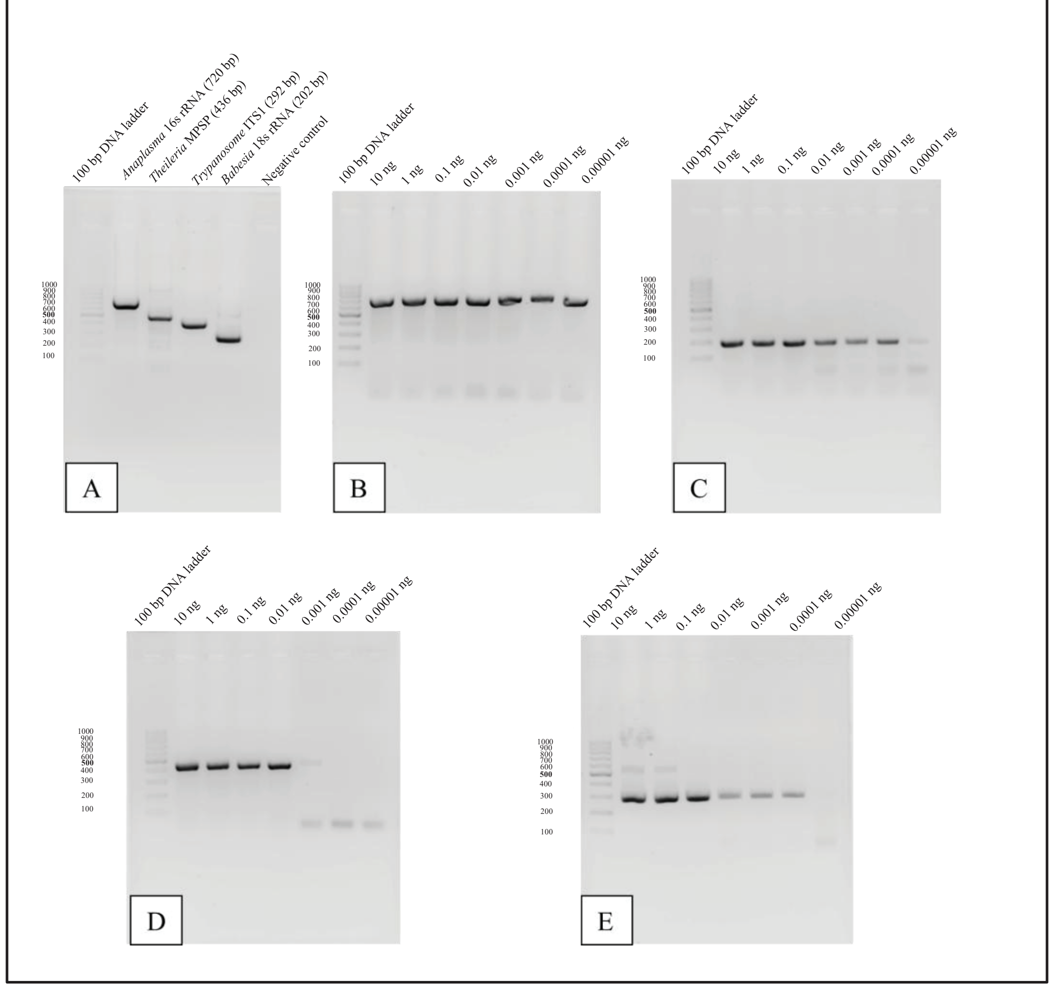

Fig. 2. Specificity (A) and sensitivity (B–E) of multiplex PCR. The PCR product of the *Anaplasma* 16s rRNA gene (B), *Babesia* 18s rRNA gene (C), *Theileria* MPSP gene (D), and *Trypanosome* ITS1 gene (E).

Table 2. Hemoparasite detection of 60 bovine blood samples using microscopic examination, single PCR, and multiplex PCR.

Pathogens	**Microscopic examination**	**Single PCR**	**Multiplex PCR**
Single infection			
Anaplasma spp.	17 (28.33%)	not detect	1 (1.67%)
Babesia spp.	not detect	11 (18.33%)	11 (18.33%)
Theileria spp.	13 (21.66%)	not detect	4 (6.67%)
Trypanosoma evansi	not detect	not detect	not detect
Total	30 (50.00%)	11 (18.33%)	16 (26.67%)
Co-infection			
Double infection			
Anaplasma and *Babesia* spp.	3 (5.00%)	14 (23.33%)	1 (1.67%)
Anaplasma and *Theileria* spp.	17 (28.33%)	not detect	not detect
Anaplasma spp. and *T. evansi*	2 (3.33%)	not detect	not detect
Babesia and *Theileria* spp.	1 (1.67%)	12 (20.00%)	15 (25.00%)
Babesia spp. and *T. evansi*	not detect	not detect	not detect
Theileria spp. and *T. evansi*	not detect	not detect	not detect
Triple infection			
Anaplasma, *Babesia*, and *Theileria* spp.	1 (1.67%)	17 (28.33%)	10 (16.67%)
Anaplasma spp., *Babesia* spp., and *T. evansi*	not detect	1 (1.67%)	not detect
Anaplasma spp., *Theileria* spp., and *T. evansi*	not detect	not detect	not detect
Babesia spp., *Theileria* spp., and *T. evansi*	not detect	2 (3.33%)	not detect
Quadruple infection			
Anaplasma spp., *Babesia* spp., *Theileria* spp., and *T. evansi*	not detect	3 (5.00%)	4 (6.67%)
Total	24 (40.00%)	49 (81.67%)	30 (50.00%)

showed double infection, which were 3 (5.00%), 17 (28.33%), 2 (3.33%), and 1 (1.67%) of *Anaplasma* spp. and *Babesia* spp., *Anaplasma* spp. and *Theileria* spp., *Anaplasma* spp. and *T. evansi*, and *Babesia* spp. and *Theileria* spp., respectively. Only one triple infection (1.67%), *Anaplasma* spp., *Babesia* spp., and *Theileria* spp., was observed. For molecular detection, 11 out of 60 (18.33%) showed single infection of *Babesia* spp. from single PCR, while multiplex PCR showed 1 out of 60 (1.67%) of *Anaplasma* spp., 11 out of 60 (18.33%) of *Babesia* spp., and 4 out of 60 (6.67%) of *Theileria* spp. Single and multiplex PCR results showed coinfection with double-, triple-, and quadruple hemoparasite infections. For double infection, single and multiplex PCR showed 14 (23.33%) and 1 (1.67%) of *Anaplasma* spp. and *Babesia* spp., respectively, and 12 (20.00%) and 15 (25.00%) of *Babesia* spp. and *Theileria* spp. Single PCR showed triple infection with *Anaplasma* spp., *Babesia* spp., and *Theileria* spp. (17/60, 28.33%), *Anaplasma* spp., *Babesia* spp., and *T. evansi* (1/60, 1.67%), and *Babesia* spp., *Theileria* spp., and *T. evansi* 2 (3.33%). Three samples (50.00%) showed quadruple infection. Multiplex PCR showed that 10 out of 60 (16.67%) patients had triple infection with *Anaplasma* spp., *Babesia* spp., and *Theileria* spp., and 4 out of 60 (6.67%) had quadruple infection.

Comparative analysis of multiplex PCR with microscopic and single PCR methods

Tables 3 and 4 show the sensitivity, specificity, PPV, NPV, and Cohen's Kappa coefficient of the multiplex PCR results compared with microscopic examination and single PCR, respectively.

For multiplex PCR and microscopic examination comparison, triple infection, *Babesia* spp., *Theileria* spp., and *T. evansi*, showed the highest sensitivity (50.0%), with 100% specificity and PPV, 98% NPV, and Cohen's Kappa value of 0.659 with substantial agreement. Single infection (*T. evansi*), double infection (*Anaplasma* spp. and *Theileria* spp., *Anaplasma* spp. and *T. evansi*, *Babesia* spp. and *T. evansi*, and *Theileria* spp. and *T. evansi*), and triple infection (*Anaplasma* spp. and *Babesia* spp. and *T. evansi*, *Anaplasma* spp. and *Theileria* spp. and *T. evansi*, and *Babesia* spp. and *Theileria* spp. and *T. evansi*) showed 100% specificity. The highest PPV (100.0%) was observed in *Babesia* spp., *Theileria* spp., and *T. evansi*, whereas *T. evansi*, *Anaplasma* spp., and *T. evansi*, and *Babesia* spp., and *T. evansi* showed 100% NPV.

Table 3. Sensitivity, specificity, PPV, NPV, and Cohen's kappa coefficient of multiplex PCR when using microscopic examination as a reference test.

Pathogens	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Cohen's kappa coefficient	
					Kappa Value	Judgment
Anaplasma spp.	0.0	98.0	0.0	76.0	−0.032	No agreement
Babesia spp.	27.0	84.0	27.0	84.0	0.109	Slight agreement
Theileria spp.	17.0	96.0	50.0	82.0	0.167	Slight agreement
Trypanosoma evansi	NaN	100.0	NaN	100.0	NaN	NA
Anaplasma and *Babesia* spp.	0.0	98.0	0.0	76.0	−0.032	No agreement
Anaplasma and *Theileria* spp.	0.0	100.0	NaN	68.0	0.0	Slight agreement
Anaplasma spp. and *T. evansi*	NaN	100.0	NaN	100.0	NaN	NA
Anaplasma, *Babesia*, and *Theileria* spp.	18.0	86.0	33.0	73.0	0.043	Slight agreement
Anaplasma spp., *Babesia* spp., and *T. evansi*	0.0	100.0	NaN	98.0	0.0	Slight agreement
Anaplasma spp., *Theileria* spp., and *T. evansi*	0.0	100.0	NaN	95.0	0.0	Slight agreement
Anaplasma spp., *Babesia* spp., *Theileria* spp., and *T. evansi*	33.0	96.0	33.0	96.0	0.298	Fair agreement
Babesia and *Theileria* spp.	33.0	79.0	29.0	83.0	0.118	Slight agreement
Babesia spp. and *T. evansi*	NaN	100.0	NaN	100.0	NaN	NA
Babesia spp., *Theileria* spp., and *T. evansi*	50.0	100.0	100.0	98.0	0.659	Substantial agreement
Theileria spp. and *T. evansi*	0.0	100.0	NaN	97.0	0.0	Slight agreement

Remark: NaN is not a number (indeterminate outcome from 0/0). NA is not available.

Table 4. Sensitivity, specificity, PPV, NPV, and Cohen's kappa coefficient of multiplex PCR when using single PCR as a reference test.

Pathogens	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Cohen's kappa coefficient	
					Kappa value	Judgment
Anaplasma sp.	NaN	98	0	100	0	Slight agreement
Babesia sp.	27	84	27	84	0.109	Slight agreement
Theileria sp.	NaN	93	0	100	0	Slight agreement
Trypanosoma evansi	NaN	100	NaN	100	NaN	NA
Anaplasma sp. and *Babesia* sp.	0	98	0	76	−0.032	No agreement
Anaplasma sp. and *Theileria* sp.	NaN	100	NaN	100	NaN	NA
Anaplasma sp. and *T. evansi*	NaN	100	NaN	100	NaN	NA
Anaplasma sp., *Babesia* sp., and *Theileria* sp.	18	84	30	72	0.016	Slight agreement
Anaplasma sp., *Babesia* sp., and *T. evansi*	0	100	NaN	98	0	Slight agreement
Anaplasma sp., *Theileria* sp., and *T. evansi*	NaN	100	NaN	100	NaN	NA
Anaplasma sp., *Babesia* sp., *Theileria* sp., and *T. evansi*	33	96	33	96	0.298	Fair agreement
Babesia sp. and *Theileria* sp.	42	79	33	84	0.190	Slight agreement
Babesia sp. and *T. evansi*	NaN	100	NaN	100	NaN	NA
Babesia sp., *Theileria* sp., and *T. evansi*	50	100	100	98	0.659	Substantial agreement
Theileria sp. and *T. evansi*	NaN	100	NaN	100	NaN	NA

Remark: NaN is not a number (indeterminate outcome from 0/0). NA is not available.

For multiplex PCR and single PCR comparison, the highest sensitivity (50.0%) was observed in triple infection (*Babesia* spp., *Theileria* spp., and *T. evansi*) with 100% specificity and PPV, 98% NPV, and substantial agreement indicated by a Cohen's Kappa value of 65.9. Notably, the highest specificity (100.0%) was found in the same samples as found in the comparison of multiplex PCR and microscopic examination. *Babesia* spp., *Theileria* spp., and *T. evansi* also showed the highest PPV (100.0%). The highest NPV (100.0%) was found in single infection (*Anaplasma* spp., *Theileria* spp., and *T. evansi*), double infection (*Anaplasma* spp. and *Theileria* spp., *Anaplasma* spp. and *T. evansi*, *Babesia* spp. and *T. evansi*, and *Theileria* spp. and *T. evansi*), and triple infection (*Anaplasma* spp. and *Theileria* spp. and *T. evansi*). In addition, the quadruple infection showed fair agreement (Cohen's kappa value of 29.8) between the two comparisons.

Positive PCR products were randomly selected and sequenced. Sequencing revealed that the *Anaplasma* 16s rRNA gene shared 99.86% sequence identity with the *Anaplasma marginale* 16S ribosomal RNA (accession no. MH020201.1). The *Theileria MPSP* gene shared 99.31% sequence identity with *T. orientalis* major piroplasm surface protein (accession no. JX648208.1), the *Trypanosome ITS1* gene shared 100.00% sequence identity with *ITS1* (accession no. MT225627.1), while the *Babesia* 18s rRNA gene shared 99.50%, 99.50%, and 99.00% sequence identities with *Cytauxzoon* sp. 18S ribosomal RNA (accession no. KT361079.1), *Theileria* sp. small subunit ribosomal RNA (accession no. OP023828.1), and *B. microti* 18S ribosomal RNA (accession no. AB243680.1), respectively.

Discussion

Blood parasites in cattle, such as *Anaplasma* spp., *Piroplasma* (*Babesia* spp. *and Theileria* spp.), and *T. evansi*, affect livestock health and production in tropical and subtropical areas (Eskezia, 2016; Jirapattharasate *et al.*, 2017; Koonyosying *et al.*, 2022; Suwan *et al.*, 2023). The gold standard for the detection of these pathogens is microscopic examination, which requires expert observation and may be misinterpreted due to subjective observations or co-infections (Kumar *et al.*, 2021). PCR-based diagnosis has been introduced with sensitivity and specificity via species-specific primers such as *16S rRNA*, *major surface proteins* (*msp*), and *groEL* for *Anaplasma* spp. (Junsiri *et al.*, 2020; Nantiya *et al.*, 2020; Seerintra *et al.*, 2023; Teja *et al.*, 2023), *18S rRNA*, *ITS*, and *merozoite surface antigens* for *Babesia* spp. (Iseki *et al.*, 2010; Liu *et al.*, 2014; Calchi *et al.*, 2024), *18s rRNA*, *msp*, and *MPSP* for *Theileria* spp. (Kaewhom and Srikijkasemwat, 2022; Rakwong *et al.*, 2022), and *ITS-1*, *Ro Tat 1.2 VSG*, and *ESAG6/7* for *T. evansi* (Dyah *et al.*, 2015; Suwan *et al.*, 2023). However, the increase in vector-borne diseases and coinfections has been reported in the Southeast Asian region, leading to the requirement of multiple PCR diagnoses (Kumar *et al.*, 2021; Koonyosying *et al.*, 2022).

Co-infections of hemoparasites in livestock have been reported, such as double infections of *Anaplasma* spp. and *Babesia* spp. (Canever *et al.*, 2014; Pradeep *et al.*, 2019; Parodi *et al.*, 2021), *Piroplasma* (*Babesia* spp. *and Theileria* spp.) (Jirapattharasate *et al.*, 2016; Silveira *et al.*, 2016; Nahal and Ben Said, 2024; Seerintra *et al.*, 2024; Saad *et al.*, 2025), triple infections of *Anaplasma* spp. and *Piroplasma* (Bilgiç *et al.*, 2013; Zhou *et al.*, 2016; Jirapattharasate *et al.*, 2017; Kundave *et al.*, 2018; Zhou *et al.*, 2019; Kumar *et al.*, 2021; Koonyosying *et al.*, 2022; Adjou Moumouni *et al.*, 2023) and *Babesia* spp., *Theileria* spp. and *T. evansi* (Charaya *et al.*, 2021), quadruple infections of *Theileria* spp., *Babesia* spp., *T. evansi*. and *Setaria* sp., and quintuple infections of *A. marginale*, *T. evansi*, *B. bovis*, *B. bigemina* and *Theileria* spp. (Ananyutthawongse *et al.*, 1999) and *Trypanosoma* spp., Microfilariae, *Anaplasma* spp., *Babesia* spp., and *Theileria* spp. (Bohman *et al.*, 2024). However, most reports have relied on microscopic examination and single- or nested PCR.

Multiplex PCR have been employed for multiple detection with various sensitivity and specificity, such as Parodi *et al.* (2021) using species-specific gene primers, *rap-1a* for *B. bovis* and *B. bigemina*, and *msp-5* for *A. marginale*, with 94.2% and 97.1%, and 95.2% and 92.7% of sensitivity and specificity for *Babesia* sp. and *A. marginale* detections. Kundave *et al.* (2018) demonstrated multiplex PCR using a set of primers, *Tams1*, *18S rRNA*, and *16S rRNA* for *T. annulata*, *B. bigemina*, and *A. marginale* with a limit of detection 0.1, 10, and 0.1 pg, respectively. While Bilgiç *et al.* (2013) used a set of primers, *cytochrome b*, *msp-1b* and *VESA–1a* for *T. annulata*, *A. marginale*, and *B. bovis* with a limit of detection of 10^{-8}, 10^{-7}, and 10^{-5} DNA template dilutions, respectively. In Thailand, multiplex PCR for *A. marginale*, *T. evansi*, *B. bovis*, *B. bigemina*, and *Theileria* sp. detections have been developed by Ananyutthawongse *et al.* (1999) using a set of primers, *msp* for *A. marginale*, repetitive nucleotide sequences for *T. evansi*, *carbamoyl phosphate synthetase II* for *B. bovis*, and *small subunit ribosomal RNA* for *B. bigemina* and *Theileria* spp., with a limit of detection of 10 pg of parasite DNA, except *T. evansi* was able to detect at 1 pg of parasite DNA.

In this study, multiplex PCR for quadruple infections, Piroplasma (*Babesia* spp. and *Theileria* spp.), *Anaplasma* spp., and *T. evansi*, were developed using *18S rRNA*, *MPSP*, *16s rRNA*, and *ITS1* primers, respectively. This multiplex PCR showed a limit of detection of 0.01 pg of *Anaplasma* and *Babesia* DNA, 0.1 pg of *Theileria* DNA, and 10 pg of *T. evansi* DNA. DNA sequencing revealed that randomly selected PCR products belonged to *A. marginale*, *T. orientalis, and T. evansi* when using *16s rRNA*, *MPSP, and ITS1* primers, respectively. Unfortunately, the PCR product of *the 18s*

rRNA primer showed conserved *18s rRNA* sequences among piroplasma species, including *Cytauxzoon* sp., *Theileria* sp., and *Babesia microti*. According to the results, *16s rRNA*, *MPSP*, and *ITS1* primers can detect blood parasites, including *Anaplasma* spp., *Theileria* spp., and *T. evansi*. The *18s rRNA* primer could be used for Piroplasma detection, such as *Babesia* spp., *Theileria* spp., and *Cytauxzoon* sp., in which this gene is highly conserved and commonly used for *Babesia* spp. detection with accurate diagnosis, but it is difficult to distinguish among Piroplasma when the amplified DNA fragment is small (Calchi *et al.*, 2024). The piroplasm can be classified by small subunit ribosomal RNA and MPSP sequencing (Özübek and Aktaş, 2019). More than 11 distinct *T. orientalis* genotypes have been identified by MPSP sequences, of which types 1 (Chitose) and 2 (Ikeda) are associated with high theileriosis morbidity and mortality in cattle (Gebrekidan *et al.*, 2020). In this study, we used the MPSP primer to distinguish between *Babesia* spp. and *Theileria* spp. and found 34 out of 60 (56.67%) and 33 out of 60 (55.00%) from single and multiplex PCR of *Theileria* infection, respectively. In addition, the *MPSP* could be used not only for detection but also to provide virulence and clinical-associated information in different *T. orientalis* strains (Seerintra *et al.*, 2024). The multiplex PCR assay developed in this study can detect the presence of 0.01–10 pg of parasite DNA. This method may be helpful for hemoparasite screening in cattle by minimizing time, cost, and labor consumption. It can also be used for veterinary blood parasite monitoring to prevent and control parasites. It can also be combined with farmer education, good hygiene, and environmental management.

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Conflict of interest

The authors declare no conflicts of interest.

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Authors' contributions

All authors contributed to the study conception and design. Conceptualization: Eukote Suwan and Sathaporn Jittapalapong; Data curation: Eukote Suwan, Piangjai Chalermwong, Preeyanuch Thongpoo, Jiravich Methawiroon, Ornarin Boonjint, Piangdow Chiawvitkan, Sakulchit Wichainchot, and Chanapath Thabthimsri; formal analysis: Eukote Suwan, Piangjai Chalermwong, Ornarin Boonjint, and Piangdow Chiawvitkan; Investigation: Eukote Suwan, Preeyanuch Thongpoo, Jiravich Methawiroon, Piangjai Chalermwong, Ornarin Boonjint, Piangdow Chiawvitkan, Sakulchit Wichainchot, and Chanapath Thabthimsri; methodology: Eukote Suwan, Piangjai Chalermwong, Preeyanuch Thongpoo and Sathaporn Jittapalapong; project administration: Ketsarin Kamyingkird and Sathaporn Jittapalapong; resources: Ketsarin Kamyingkird and Sathaporn Jittapalapong; software: Eukote Suwan and Piangjai Chalermwong; validation: Eukote Suwan, Jiravich Methawiroon, Piangjai Chalermwong, Ornarin Boonjint, Piangdow Chiawvitkan, and Preeyanuch Thongpoo; writing - original draft: Eukote Suwan; writing-review and editing: Eukote Suwan, Piangjai Chalermwong, Preeyanuch Thongpoo, Jiravich Methawiroon, Ornarin Boonjint, Piangdow Chiawvitkan, Sakulchit Wichainchot, and Chanapath Thabthimsri, Ketsarin Kamyingkird and Sathaporn Jittapalapong.All authors have read and approved the published version of the manuscript.

Data availability

Data sets generated during and/or analyzed during the current study are available upon reasonable request from the corresponding author.

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