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RESEARCH

Molecular occurrence and genetic identification of *Babesia* spp. and *Theileria* spp. in naturally infected cattle from Thailand

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Abstract

Piroplasm including *Babesia* spp. and *Theileria* spp. in cattle can cause illness that affects livestock productivity, resulting in significant production losses, especially in tropical and subtropical regions such as Thailand. This study aimed to investigate the prevalence of bovine piroplasms and to identify these blood parasites based on the *18S ribosomal RNA* gene in cattle in the northeastern part of Thailand. Piroplasmid infections among beef and dairy cattle were examined using nested PCR. Furthermore, amplicon DNA was sequenced and analyzed, and a phylogenetic tree was constructed to determine the genetic diversity and relationships of the parasite in each area. A total of 141 out of 215 (65.6%) cattle were positive for infection with *Babesia* or *Theileria*. DNA analysis revealed that infection by *Babesia bigemina*, *Babesia bovis*, *Theileria orientalis*, *Theileria sinensis*, and *Theileria* sp. were common piroplasms in cattle in this region, with a high sequence shared identity and similarity with each other and clustered with isolates from other countries. This study provides information on the molecular epidemiology and genetic identification of *Babesia* spp. and *Theileria* spp. in beef and dairy cattle to provide a better understanding of piroplasm infection in cattle in this region, which will help control these blood parasites. Moreover, this is the first report identifying *T. sinensis* circulating among Thai cattle.

Keywords Babesiosis · Theileriosis · Bovine · Molecular prevalence · Piroplasm

Introduction

In animal production, the emergence and spread of piroplasmosis caused by piroplasm have gained prominence as a significant threat that poses constant challenges to livestock health and productivity. Piroplasms are protozoan parasites belonging to phylum Apicomplexa, order Piroplasmida, and genera *Babesia* and *Theileria*, which have a complex life cycle that involves transmission through ticks and an obligate intracellular stage within the host's blood cells, resulting in hematologic disorders (Uilenberg 2006; Lempereur et al. 2017; Jalovecka et al. 2018). In cattle, the *Babesia* parasites enter the bloodstream and invade their red blood cells primarily manifesting as hemolytic anemia, fever, jaundice, and hemoglobinuria (Hashem et al. 2018), while *Theileria* parasites enter and invade white blood cells, particularly leukocytes, and exceed in the erythrocytes. *Theileria* infection frequently presents as mild fever, hemorrhages on the mucosa and serosal surfaces, anemia, jaundice, and lymphocyte enlargement (Ma et al. 2020). Cattle become infected with *Babesia* or *Theileria* when they are bitten by infected ticks, predominantly ticks in the genus *Hyalomma* spp. (Aktas et al. 2004; Sang et al. 2021), *Rhipicephalu*s spp. (Giglioti et al. 2018; Kakati et al 2015), and *Haemaphysalis* spp. (Marendy et al. 2020; Phipps et al. 2022).

Previously, *Babesia* and *Theileria* infections in cattle have been examined based on the morphological characteristics of these parasites using blood smears under a microscope. However, molecular techniques have assisted and

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facilitated more accurate species identification, along with the assessment of genetic diversity in these piroplasms. In previous studies in Thailand, molecular detection of piroplasms in cattle indicated the presence of endemic species, including *Babesia bovis*, *Babesia bigemina* (Cao et al. 2012; Simking et al. 2013; Srionrod et al. 2022), and *Theileria orientalis* (Jirapattharasate et al. 2016, 2017; Adjou Moumouni et al. 2023). For molecular detection, the small subunit ribosomal RNA gene (*18S rRNA* gene) is one of the most genes effectively utilized for parasite identification and also phylogenetic analysis of piroplasmids (Allsopp and Allsopp 2006; Lack et al. 2012; Kumar et al. 2022).

However, information on the prevalence of *Babesia* and *Theileria* infections in cattle, as well as inadequate information on their genetic characterization which is essential for the control and prevention of these piroplasms, is limited in Thailand and needs updating. In the present study, we demonstrated the molecular screening of *Babesia* or *Theileria* infection in beef and dairy cattle from the northeastern part of Thailand. We also identified the *Babesia* and *Theileria* species and conducted a phylogenetic analysis based on the *18S rRNA* gene. This epidemiological study investigated the species and genetic diversity of *Babesia* and *Theileria* in cattle to enhance the understanding of these infections and to raise awareness among farmers and healthcare professionals for effective strategic control.

Materials and methods

Study area and sample collection

A comprehensive investigation was conducted from July 2021 to July 2023 in six provinces in the northeastern part of Thailand: Udon Thani, Khon Kaen, Maha Sarakham, Roi Et, Ubon Ratchathani, and Chaiyaphum (Fig. 1). A total of 215 blood samples were collected from bovine species, comprising 134 samples from beef cattle and 81 samples from dairy cattle. Blood samples of approximately 1–2 mL were collected from the jugular vein or coccygeal vein and preserved in ethylenediaminetetraacetic acid (EDTA) anticoagulant tubes. Information regarding the sex, age, and production type of individual samples was systematically recorded and cataloged as part of the dataset under investigation. Blood samples were transported on ice to the laboratory at the Faculty of Veterinary Sciences of Mahasarakham University and stored at − 20 ℃ for long-term preservation until DNA extraction. All blood samples taken

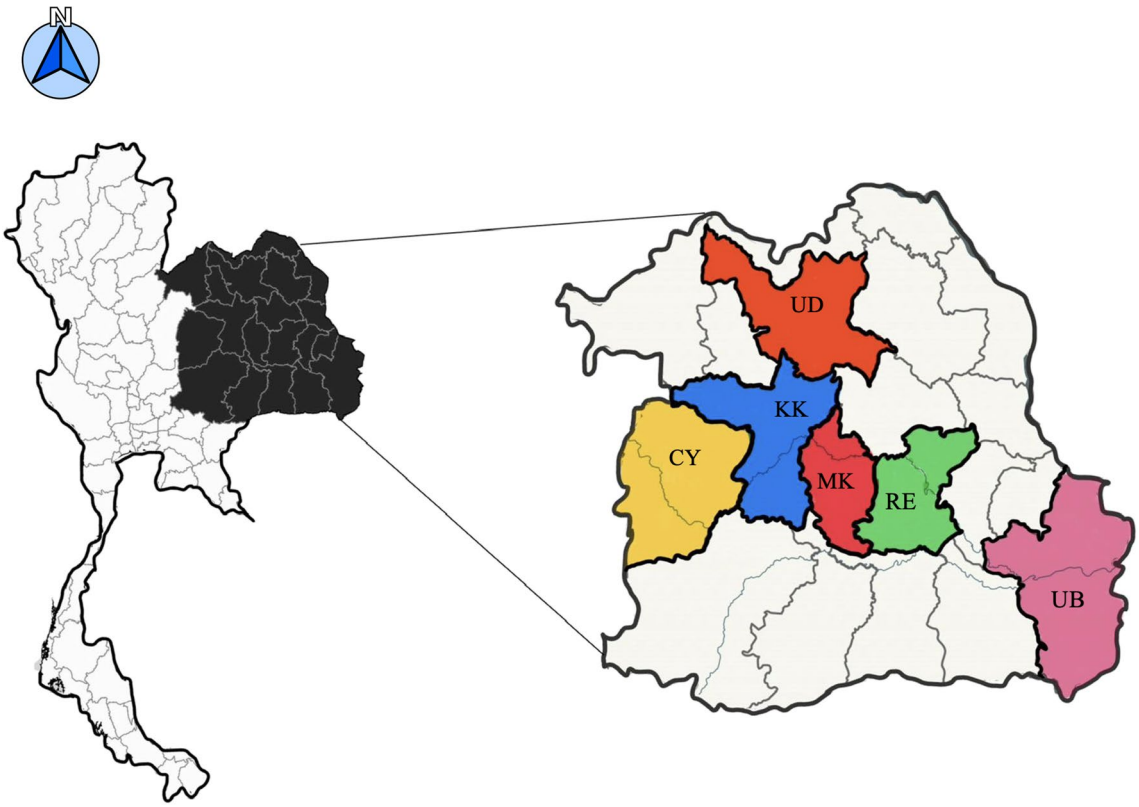

Fig. 1 Map of the study area where cattle blood samples were collected across six provinces in the northeastern part of Thailand, consisting of Udon Thani (UD), Khon Kaen (KK), Chaiyaphum (CY), Maha Sarakham (MK), Roi Et (RE), and Ubon Rachathani (UB)

were approved by their owners and received approval from the ethics committee.

DNA extraction and nested PCR amplification of *Babesia* and *Theileria 18S rRNA* gene

Isolation of DNA from the collected blood samples used the GF-1 blood DNA extraction kit procedure (Vivatis, Malaysia), adhering to standardized protocols as prescribed by the manufacturer. Each extracted DNA sample underwent examination for *Babesia* and *Theileria* infection utilizing a nested PCR method. This method employed specific primers designed to target the *18S rRNA* gene, approximately 1500 bp in length, of the parasite, as previously described (Masatani et al. 2017). The first PCR step utilized the primer pair, namely, BTH 18S 1st F (5′-GTGAAACTGCGAATGGCTCATTAC-3′) and BTH 18S 1st R (5′-AAGTGATAAGGTTCACAAAACTTCCC-3′), while the second step utilized the primer pair BTH 18S 2nd F (5′-GGCTCATTACAACAGTTATAGTTTATTTG-3′) and BTH 18S 2nd R (5′-CGGTCCGAATAATTCACCGGAT-3′). This nested PCR approach enabled the amplification of DNA from protozoa in the genera *Babesia*, *Theileria*, and *Hepatozoon*.

The nested PCR reactions were conducted in a final volume of 25 µL. Each reaction mixture comprised of 0.4 µM of each primer, 1 U of *Taq* polymerase (Vivatis, Malaysia), 1×PCR buffer, 1.5 mM $MgSO_4$, 0.2 mM dNTPs, and 2 µL of template DNA (extracted DNA for the first PCR and PCR product from the primary amplification for the second PCR). Both PCR reactions comprised 35 cycles, which included denaturation at 95 °C for 45 s, annealing at 50 °C for 45 s, and extension at 72 °C for 90 s, followed by a final extension step at 72 °C for 5 min. Negative controls were prepared using PCR master mixes containing only the primers without any DNA template. PCR amplification was performed using a thermal cycler (Biometra GmbH, Germany). The resulting approximately 1500 base pairs from nested PCR were then visualized using 1% agarose gels stained with ViSafe Red Gel Stain (Vivantis, Malaysia) and examined under ultraviolet light using a gel documentation system from Bio-Rad, USA.

Descriptive statistics were employed to summarize the prevalence of *Babesia* or *Theileria* infection. Chi-squared tests were then employed to analyze the associations between infection rates and host factors, including breed, sex, and age with a significance level of *p*-value less than 0.05 (Social Science Statistics 2023).

DNA sequencing and phylogenetic analysis

We randomly selected 65 PCR amplicons from dispersed sampling sites, representing 50% of positive samples from each site, for purification and direct sequencing. The PCR products targeting the *18S rRNA* genes underwent purification and sequencing at a commercial sequencing facility (1st Base, Malaysia). Electrograms of the sequences were meticulously examined for quality, appropriate length, and absence of double or multiple nucleotide peaks. The obtained DNA sequences were aligned and trimmed using the BioEdit sequence alignment editor program (Hall 1999). Subsequently, the nucleotide sequences were analyzed for similarity to sequences in the GenBank database using the BLAST program hosted by NCBI (https://www.ncbi.nlm.nih.gov/). Haplotype identification from the *18S rRNA* sequences of *Babesia* and *Theileria* was conducted using the DnaSP6 program (Rozas et al. 2017).

The obtained sequences of the partial *18S rRNA* gene of *Babesia* and *Theileria* in this study were approximately 1405 bp in length (ranging from 1343 to 1458 bp). The resulting partial *18S rRNA* gene sequences represented each *Babesia* and *Theileria* haplotype were then deposited into the GenBank database with accession numbers PP380178–PP380189. Phylogenetic relationships among the *18S rRNA* haplotypes from this study and 27 related sequences from various geographical locations in GenBank were inferred using the maximum likelihood method in MEGA X (Kumar et al. 2018). Bootstrap analysis with 1000 replications was employed to assess the confidence of branching patterns in the trees.

Results

Prevalence of *Babesia* and *Theileria* in cattle

From a total of 215 samples, comprising 180 females and 35 males, spanning an age range from 2 months to 10 years, 65.6% (141/215) exhibited infection with *Babesia* or *Theileria*, as determined by nested PCR analysis. Specifically, among females, 65% (117/180) were found to be infected, whereas among males, the infection rate was slightly higher at 68.6% (24/35). Chi-squared analysis revealed that the observed differences in infection rates between sexes were not statistically significant. Furthermore, when considering the distribution of infection across different production types, it was observed that 66.4% (89/134) of beef cattle and 64.2% (52/81) of dairy cattle were infected. Chi-squared analysis indicated no significant difference in infection rates between beef and dairy cattle. Animals within the age range of 0–1 year showed an infection rate of 52.7% (20/38), adult animals aged between 1 and 6 years displayed an infection rate of 67.1% (104/155), and old animals aged over 6 years showed an infection rate of 77.3% (17/22). Despite these observed differences in infection rates across age groups, statistical analyses did not reveal any significant age-related associations with infection susceptibility within the studied population (Table 1).

Table 1 Characteristics of cattle infected with *Babesia* sp. or *Theileria* sp

Characteristics	No. of cattle (*n*)	No. of positive infection	Prevalence (%)	95% confidence interval
Cattle types				
Beef cattle	134	89	66.4	57.8–74.3
Daily cattle	81	52	64.2	52.8–74.6
Chi-squared value (*p*-value)	0.1103 (0.74)			
Gender				
Female	180	117	65	57.6–72.0
Male	35	24	68.6	50.7–83.2
Chi-squared value (*p*-value)	0.1656 (0.68)			
Age (years)				
Calf (0–1)	38	20	52.6	35.8–69.0
Adult (1–6)	155	104	67.1	59.1–74.4
Old (> 6)	22	17	77.3	54.6–92.2
Chi-squared value (*p*-value)			4.3131 (0.12)	
Total	215	141	65.6	58.8–71.9

The *p*-value compares the differences between characteristics in each column

Babesia and *Theileria* identification

Among the positive samples, a subset of 65 PCR products (representing 50% of positive samples from each province) was randomly selected for sequencing analysis and resulted in the successful sequencing of 64 specimens. Sequencing identified the presence of *Babesia bovis*, *B. bigemina*, *Thileria* sp., *T. orientalis*, and *T. sinensis* in cattle in the northeastern part of Thailand. Among the 64 sequences, 6 corresponded to *Babesia* species (comprising 2 sequences of *B. bovis* and 4 sequences of *B. bigemina*), and 58 sequences corresponded to *Theileria* species (comprising 28 samples of *T. orientalis*, 15 samples of *Theileria* sp., and 13 samples of *T. sinensis*).

Further analysis of the sequences revealed the presence of distinct haplotypes within both *Theileria* and *Babesia* species. Specifically, 58 sequences representing *Theileria* species were classified into 6 haplotypes, with 4 haplotypes associated with *T. orientalis* (accession numbers PP380178, PP380179, PP380181, and PP380182), one haplotype was *Theileria* sp. (accession number PP380180), and one haplotype was *T. sinensis* (accession number PP380183). The remaining 6 sequences corresponded to *B. bovis* for 2 haplotypes (accession nos. PP380184 and PP380185), while *B. bigemina* was differentiated into 4 distinct haplotypes (accession nos. PP380186–PP380189). Sequencing and DNA analysis revealed that infection of cattle with the piroplasms *B. bigemina*, *B. bovis*, *T. orientalis*, *T. sinensis*, and *Theileria* sp. was common in this region, with sequence similarities ranging between 99 and 100%, and sequences were homologous with sequences from other countries (Table 2).

Phylogenetic tree

The phylogenetic analyses, utilizing the *18S rRNA* gene, delineated distinct evolutionary lineages within *Theileria* and *Babesia*. *Theileria* was observed to segregate into three primary clades: *T. orientalis*, *T. sinensis*, and *Theileria* sp. In Thailand, *T. orientalis* was found to have four haplotypes, which grouped into two subgroups. Subgroup 1 included three haplotypes (PP380179, PP380181, PP380182) closely related to *T. orientalis* from dogs in Myanmar (LC602478.1), cattle in South Korea (MT889728.1), buffalo in India (OR068053.1), cattle in China (KU363043.1), and cattle in Pakistan (MG599099.1). Subgroup 2 contained one haplotype (PP380178) closely related to *T. orientalis* from cattle in Türkiye (OR211416.1) and ticks in China (MH208641.1). For *T. sinensis*, all isolates, including those from Thai cattle (PP380183) as well as cattle from Malaysia (MT271911.1) and China (KX115427.1, KF559355.1, EU274472, HM538203.1), formed a single cohesive group. *Theileria* sp. exhibited two distinct subgroups in the phylogenetic analysis: one subgroup included isolates from cattle in India (OR067892.1), while the other subgroup consisted of isolates from Thai cattle (PP380180), which clustered together with isolates from cattle in Myanmar (LC57817.1), cattle in China (MN252454.1), and buffaloes in China (DQ286801.1). From phylogenetic analysis, *T. orientalis* and *T. sinensis* are closely related, whereas *Theileria* sp. is more distantly related and grouped with *T. annulata*.

Sequence analysis confirmed the presence of *Babesia 18S rRNA* sequences, which were categorized into two main clades: *B. bovis* and *B. bigemina.* In this study, two haplotypes of *B. bovis* from Thailand were placed into

Table 2 Haplotypes of 64 sequences of *18S rRNA* from beef and dairy cattle in Thailand and National Center for Biotechnology Information BLAST results

Haplotype	Sample IDs	NCBI GenBank accession number	Closest sequences in NCBI GenBank (% similarity)	Parasites
1	KM01, KM06, KM09, RE01, RE02, RE07, KW11, KW12, UB02, K01	PP380178	MH208641.1 (99.9) OR211416.1 (99.9) CP056070.2 (99.9)	*Theileria orientalis*
2	KM11, UD05, K06, K11, K14, PM02, UB03, UB05, KW01, KW09, KW14, CY09, CY11, CY26	PP380179	OR068053.1 (99.9) LC602478.1 (99.9) AB520956.1 (99.9)	*Theileria orientalis*
3	KM08, KM12, KM30, UD04, UB07, UB08, K04, K05, K08, K12, RE04, RE05, NC05, KW04, PM01, CY17	PP380180	OR067892.1 (100) MN252454.1 (100) DQ286801.1 (100)	*Theileria* sp.
4	UB04, MM03	PP380181	OR068050.1 (99.7) LC602478.1 (99.6) AB520956.1 (99.6)	*Theileria orientalis*
5	K02, K03	PP380182	OR068053.1 (99.9) LC602478.1 (99.9) MN252441.1 (99.9)	*Theileria orientalis*
6	UD02, UD07, KM15, KM16, PM03, KW02, KW03, KW15, KW16, CY01, CY04, CY07, CY20, CY23	PP380183	MT271911.1 (99.9) MT271902.1 (99.9) KF559355.1 (99.9)	*Theileria sinensis*
7	KM19	PP380184	MH257728.1 (99.1) MH257734.1 (99.1) KY805831.1 (99)	*Babesia bovis*
8	NC03	PP380185	MH257726.1 (99.9) MH046909.1 (99.4) CP125250.1 (99.3)	*Babesia bovis*
9	NC01	PP380186	AY603402.1 (99.9) MH208614.1 (99.9) KY805825.1 (99.9)	*Babesia bigemina*
10	NC02	PP380187	AY603402.1 (99.9) KY805825.1 (99.8) MH257700.1 (99.8)	*Babesia bigemina*
11	NC06	PP380188	KP710227.1 (99.8) OP604189.1 (99.8) JX495402.1 (99.8)	*Babesia bigemina*
12	CY14	PP380189	OP604193.1 (99.6) KP710227.1 (99.5) JX495402.1 (99.5)	*Babesia bigemina*

different subgroups: the first haplotype (PP380184) grouped with *B. bovis* isolates from cattle in China (KY805831.1) in subgroup 1, while the second haplotype (PP380185) grouped with *B. bovis* isolates from cattle in South Africa (MH257728.1), Brazil (EF458212.1), and the USA (L31922) in subgroup 2. Additionally, four haplotypes of *B. bigemina* from Thailand were classified into two subgroups: the first haplotype (PP380189) clustered with *B. bigemina* isolates from ticks in Taiwan (OP604193.1) and cattle in China (JX495402.1), while the remaining haplotypes (PP380186–PP380188) formed a separate cohesive group (Fig. 2).

Discussion

Epidemiology research efforts are needed to enhance our understanding of bovine babesiosis and theileriosis and to develop effective strategies for disease control and prevention in Thai cattle populations. In the present study, we

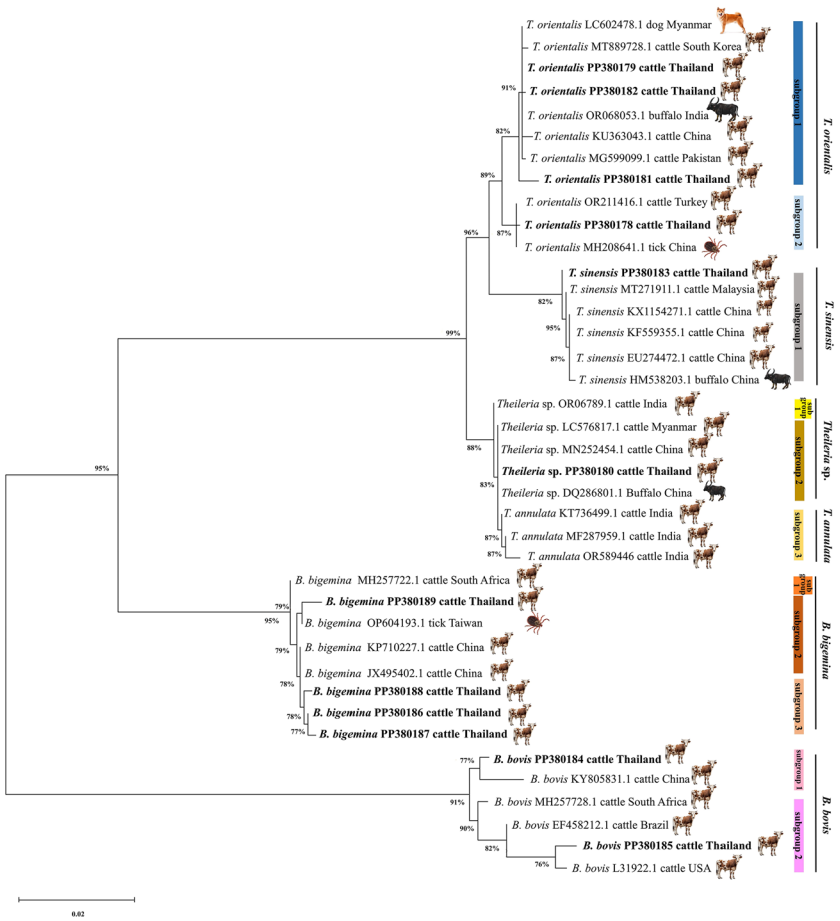

Fig. 2 Phylogenetic analyses of *Babesia* and *Theileria 18S rRNA* sequences obtained from Thailand cattle and related sequences in GenBank using the maximum likelihood method. The sequences determined in this study are shown in bold font and the percentage of trees in which associated taxa clustered together is shown next to the branch

demonstrated the molecular detection based on *18S rRNA* gene in samples from beef and dairy cattle in six provinces in the northeastern part of Thailand. This study documented the highest prevalence of piroplasmid infection (*Babesia* or *Theileria*) reported in Thailand to date at approximately 65.6% (95% CI: 58.8–71.9%). Furthermore, there was considerable variation in infection rates observed across sampled farms, ranging from 35 to 92.3%. This variability may be attributed to multiple factors, including herd size and farm management practices, particularly those about tick control strategies (Muhanguzi et al. 2010). In comparison to previous reports, which indicated prevalence rates of *B. bovis* and *B. bigemina* in cattle of 12% and 21% respectively in 2012 (Cao et al. 2012) and 11.1% and 12.5% respectively in 2017 (Jirapattharasate et al. 2017), this study demonstrates a substantial increase. Notably, recent studies in 2022–2023 have shown a decrease in *Babesia* prevalence, ranging from 1.2 to 5.8% (Koonyosying et al. 2022; Srionrod et al. 2022; Adjou Moumouni et al. 2023). Regarding *Theileria* prevalence, earlier research in 2017 reported a prevalence rate of 7.8% (Jirapattharasate et al. 2017), whereas a study conducted in 2022 recorded a higher prevalence of 36.5% (Koonyosying et al. 2022). *Babesia* and *Theileria* have a worldwide distributed with molecular prevalence of 25.3% in selected areas of China and Pakistan (Hassan et al. 2018), 36.1% in Kyrgyzstan (Aktaş et al. 2019), 52.8% in Nepal (Dhakal et al. 2023), and 87.3% in Nigeria (Famuyide et al. 2020). The varying prevalence in each regions underscores the influence of climatic and meteorological conditions which influence tick vector populations (M'ghirbi et al. 2008).

Through DNA sequencing analysis, our study identified *T. orientalis* as the dominant species of piroplasm, followed by *Theileria* sp., *T. sinensis*, *B. bigemina*, and *B. bovis* in cattle farms in Thailand. This observation is consistent with prior findings in central and northern Thailand, which also highlighted the prevalence of *T. orientalis* as the dominant species (Koonyosying et al. 2022). Furthermore, similar dominance of *Theileria* has been reported in other regions such as China (Zhou et al. 2019) and Kyrgyzstan (Aktaş et al. 2019). *T. orientalis* was classified as a non-transforming species and grouped among lower pathogenic organisms (Sivakumar et al. 2014). Classification of this piroplasm is typically based on sequencing of the small subunit ribosomal RNA and the major piroplasm surface protein (MPSP) (Özübek and Aktaş, 2019). To date, researchers

have identified at least 11 distinct genotypes within the *T. orientalis* worldwide based on the MPSP sequence. Among these genotypes, types 1 (Chitose) and 2 (Ikeda) are notable for causing clinical oriental theileriosis in cattle, with high morbidity and mortality (Gebrekidan et al. 2020). For future investigations, there is a significant opportunity to focus on the MPSP gene which will provide insights into the virulence and clinical outcomes associated with different strains of *T. orientalis*. In addition, production type, age, and sex did not exert a significant influence on the likelihood of infection with *Babesia* or *Theileria* in the studied cattle population. This finding correlated with previous studies in Malaysia and Egypt which similarly demonstrated that sex was not correlated with infection. However, factors such as production type and age exhibited significant associations ($p < 0.05$) with the prevalence of *T. orientalis* (Ola-Fadunsin et al. 2020; Selim et al. 2022).

Previous studies reported that the *18S rRNA* fragments are appropriate markers to determine the genetic diversity for blood parasites (Bawm et al. 2021; Nehra et al. 2022). In this study, analysis of *18S rRNA* revealed a notable degree of sequence similarity within the *Babesia* and *Theileria* of this population and the GenBank database. Phylogenetic analysis showed that *Theileria* could be divided into three groups: *T. orientalis*, *T. sinensis*, and *Theileria* sp. Furthermore, our investigation identified *T. orientalis* and *T. sinensis* as genetically more similar to each other, forming a distinct cluster separated from *Theileria* sp. suggesting a closer evolutionary relationship between *T. orientalis* and *T. sinensis*, distinguishing them from *Theileria* sp. based on *18S rRNA* gene. Previously, epidemiological data indicated the presence of *T. orientalis* and *Theileria* sp. in Thailand, while *T. sinensis* had not been documented in the country. This study has now confirmed the first presence of *T. sinensis* circulating within the cattle population in Thailand. In addition, *T. sinensis* is generally considered to have low pathogenicity compared to other transforming species like *T. parva* or *T. annulata*. It is transmitted by *Haemaphysalis* ticks and commonly infects cattle, with reports in China and Malaysia (Kho et al. 2017; Jia et al. 2020). The discovery of this new protozoa in Thailand could be attributed to the presence of tick hosts and the tropical climate, which provide favorable conditions for the development of the protozoa's life cycle when new pathogens are introduced.

Conclusion

This study showed a notably heightened molecular prevalence of piroplasm infection in cattle in Thailand compared to preceding reports, accomplished through the utilization of primers targeting the *18S rRNA* gene. Through this investigation, advancements in our contemporary comprehension of the distribution and genetic diversity of piroplasms in both beef and dairy cattle populations in Thailand have been achieved. *T. orientalis* emerges as the dominant species, followed by *Theileria* sp., *T. sinensis*, *B. bigemina*, and *B. bovis*. The identification of *Babesia* and *Theileria* presence underscores a public health concern, indicating the potential for these animals to act as reservoirs for parasites, serve as vectors for pathogen transmission, and facilitate disease dissemination.

Author contribution Conceptualization: Tossapol Seerintra, Tongjit Thanchomnang, and Supawadee Piratae; methodology: Tossapol Seerintra, Wongwiwat Krinsoongnern, Tongjit Thanchomnang, and Supawadee Piratae; formal analysis and investigation: Tossapol Seerintra, Wongwiwat Krinsoongnern, Tongjit Thanchomnang, and Supawadee Piratae; writing—original draft preparation: Tossapol Seerintra and Supawadee Piratae; writing—review and editing: Tossapol Seerintra, Tongjit Thanchomnang, and Supawadee Piratae; funding acquisition: Supawadee Piratae; all authors read and approved the final manuscript.

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Data availability All data generated or analyzed during this study are included in this article. The newly generated sequences were deposited in the GenBank database under the accession numbers PP380178-PP380189.

Declarations

Ethical approval All experimental procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee of Mahasarakham University (IACUC-MSU-3/2023).

Consent to participate Written informed consent was obtained from the cattle owner.

Consent for publication Not applicable.

Competing interests The authors declare that they have no competing interests.

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