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Research Article

Molecular detection of Haemoparasitic *Babesia* spp. infection in Thai flighting bulls in Nakhon Si Thammarat province, Thailand

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ABSTRACT

Bovine babesiosis significantly impacts cattle health and productivity; however, its epidemiology in Thai fighting bulls remains understudied. This research investigated the prevalence and genetic characteristics of Babesia species in these bulls from Nakhon Si Thammarat province, Thailand. Blood samples from 267 bulls across 13 districts were analyzed using PCR targeting the ama-1 gene. The overall Babesia prevalence was 12.36%. District-specific infection rates were: Nophitam, 20.0% (4/20); Phipun, 15.0% (3/20); Ronphibun, 31.81% (7/22); Chulaphon, 15.0% (3/20); Chauat, 25.0% (5/20); Huasai, 25.0% (5/20); and, Chianyai, 26.09% (6/23). Sequencing identified *Babesia bovis* as the sole infecting species; *B. bigemina* was not detected. Nucleotide sequencing confirmed that all positive samples were *Babesia bovis*, and *B. bigemina* was not detected. Phylogenetic analysis of the *B. bovis* ama-1 sequences delineated two distinct genetic clades with geographical correlation. Clade I, comprising isolates from the northern districts of Nophitam and Phipun, exhibited close genetic relatedness to isolates from Brazil and other regions of Thailand. Conversely, Clade II, encompassing isolates from the southern districts of Ronphibun, Chulaphon, Chauat, Huasai, and Chianyai, showed genetic similarity to isolates from Israel and Sri Lanka. The varying district prevalence highlights heterogeneous infection distribution, indicating high-risk areas for targeted interventions. Distinct phylogenetic clades suggest regional *B. bovis* strain differences, warranting further investigation into transmission and management implications for this economically and culturally important fighting bull population.

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1. Introduction

Bovine babesiosis, a tick-borne disease caused by intraerythrocytic protozoa of the genus Babesia (phylum Apicomplexa), results in significant economic losses globally, affecting both domestic and wild bovids (Almazán *et al*., 2022). Key etiological agents include *B. bovis*, *B. bigemina*, *B. divergens*, and *B. major* (Santos *et al*., 2023). In Thailand, *B. bovis* and *B. bigemina* are the primary pathogens, with *B. bovis* associated with more severe disease, indicating higher virulence (Cao *et al*., 2012; Nagano *et al*., 2013; Srionrod *et al*., 2022). The distribution of *Babesia* spp. is closely linked to their tick vectors, particularly the *Rhipicephalus microplus* species complex, a prevalent ectoparasite of cattle in tropical and subtropical regions (Yin *et al*., 1997; Bock *et al*., 2004). *B. bovis* undergoes both sexual and asexual reproduction: the sexual phase within the tick vector and asexual reproduction within erythrocytes of the mammalian host (Bock *et al*., 1992). While ticks are the primary vectors, mechanical transmission by blood-feeding flies from the families Tabanidae and Muscidae, is also possible (Van den Bossche and Mudenge, 1999; Sontigun *et al*., 2022).

Babesia infection initiates with the invasion of erythrocytes by sporozoites, followed by asexual reproduction via binary fission. Released merozoites infect additional erythrocytes, with some differentiating into gametocytes. The sexual phase commences when a tick ingests blood containing these gametocytes (Jalovecka *et al*., 2018). Babesia replication within erythrocytes leads to hemolysis and hemolytic anemia. Hemograms of infected cattle often reveal macrocytic hypochromic anemia and thrombocytopenia (Mahmoud *et al*., 2015). Furthermore, *Babesia* spp. infection increases oxidative stress markers and enhances cell-mediated immune responses, as observed in *B. bovis*-positive cattle (Attia and Khalifa, 2023).

Tentative diagnosis of bovine babesiosis is based on clinical signs. Microscopic examination of stained blood smears remains the gold standard for confirmatory diagnosis, although it may yield false negatives in subclinical and chronic infections with low parasitemia (Fahrimal *et al*., 1992; Bal *et al*., 2016). Serological assays, while available, can exhibit cross-reactivity, limiting their ability to differentiate closely related Babesia species (Bal *et al*., 2016). Immunochromatographic test (ICT) strips are preferred for rapid field diagnosis (Guswanto *et al*., 2017; Ganzinelli *et al*., 2020). Nucleic acid-based assays, particularly polymerase chain reaction (PCR), provide high sensitivity and specificity for the identification of Babesia species (Bal *et al*., 2016; Jirapattharasate *et al*., 2017; Hossain *et al*., 2023). The ama-1 gene is a valuable target for species-specific PCR assays due to its conserved nature within species and variability between species (Torina *et al*., 2010; Sivakumar *et al*., 2012; Niu *et al*., 2015). In Thailand, sequence analysis of *B. bovis* ama-1 (Bbama-1) has revealed low polymorphism and high conservation (95.46-99.94%) among Thai and global isolates (Nagano *et al*., 2013; Rittipornlertrak *et al*., 2017). This suggests that the conserved Bbama-1 region may be suitable for detecting regional variations in *B. bovis*. Additionally, AMA-1 is considered a potential antigen for vaccine development (Rittipornlertrak *et al*., 2017).

Thai fighting bulls represent more than just livestock; they are integral to a cultural tradition that underpins a substantial economic ecosystem, particularly in southern Thailand. This thriving industry encompasses a wide network of activities, including livestock trade, specialized breeding programs focused on desirable fighting traits, and a significant demand for veterinary services catering to these high-value animals (Rakjan, 2023). Further underscores the financial significance of Thai bull fighting, which also supports livelihoods, drives local tourism, and contributes to the preservation of indigenous cattle breeds (Pramahasuriya *et al*., 2024). The investigation of *Babesia* spp. prevalence in Thai fighting bulls is of significant importance due to the cultural and substantial economic value. Despite this economic relevance, the epidemiological status of Babesia infection within this specific population remains largely uncharacterized, highlighting a critical lack of baseline data necessary for understanding the current disease burden and for the development and evaluation of effective control and prevention strategies. This study aimed to determine the prevalence of *Babesia* spp. in Thai fighting bulls in Nakhon Si Thammarat province,

Thailand. Additionally, the study sought to analyze *B. bovis* ama-1-derived regions to identify genetic variability and selection signatures.

2. Materials and Methods

2.1 Animal ethical approval

This study was conducted in accordance with protocols approved by the Institutional Animal Care and Use Committee (IACUC) of Rajamangala University of Technology Srivijaya (IAC 003-06-62).

2.2 Sample collection

Sample collection was conducted from April 2020 until May in 2021, across 13 districts in Nakhon Si Thammarat: N01 Nophitam, N02 Phipun, N03 Chawang, N04 Thungyai, N05 Nabon, N06 Changklan, N07 Lansaka, N08 Ronphibun, N09 Chulaphon, N10 Chauat, N11 Huasai, N12 Chianyai and N13 Pakphanang (Figure1). Approximately 3-5 milliliter (ml) of venous blood were collected from the jugular veins of individual male fighting bulls (age 2-5 years) using 10 ml syringes with 18G needles. The blood was dispensed into BD Vacutainer® blood collection tubes with the ethylenediaminetetraacetic acid (EDTA) anticoagulant (BD, USA) and kept on ice during transport to the laboratory for DNA extraction.

2.3 DNA extraction

Genomic DNA was extracted from all blood samples using the DNeasy Blood and Tissue Kit (Qiagen, USA) according to the manufacturer's instructions. DNA concentration was determined using a NanoDrop 2000c Spectrophotometer (Thermo Scientific, USA) at 260/280 nm. Extracted DNA was diluted to 10 ng/μL and stored at -20 °C until nested-PCR analysis.

2.4 PCR for Babesia ITS

A nested PCR assay was performed to detect *B. bovis* and *B. bigemina* using ITS-specific primers (Cao *et al*., 2012) (Table 1) in a T100 thermal cycler (Bio-Rad, USA). The amplification reaction involved two rounds using different primer sets with EmeraldAmp® GT PCR Master Mix (TaKaRa Bio Inc., Japan). Each 50 μL reaction contained 25 μL EmeraldAmp GT PCR Master Mix (2X Premix), 18 μL DNase/RNase-free deionized water, 2 μL of forward and reverse primer mix, and 5 μL of DNA template. PCR conditions were: initial denaturation at 95 °C for 3 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 60 s for both rounds of amplification. DNase/RNase-free water was used as a no-template control. Babesia DNA positive controls, Theileria and Anaplasma DNA controls provided by the Faculty of Veterinary Sciences, Rajamangala University of Technology Srivijaya, were included in all PCR assays. PCR products were electrophoresed on a 1.2% agarose gel in 1× TBE buffer for 30 min at 3 V/cm and visualized using SYBR Gold nucleic acid gel stain (Invitrogen, USA). A 100 bp DNA Ladder (Thermo Fisher, USA) was used as a molecular weight marker

2.5 PCR for Babesia ama-1 gene

To amplify the complete ama-1 gene sequence of *B. bovis*, four primer sets were designed based on the reference sequence of *B. bovis* apical membrane antigen 1 (NCBI accession number: XM_001610993) (Table 1; Rittipornlertrak *et al*., 2017). All Babesia-positive samples, as determined by ITS-PCR, underwent nested PCR using the same conditions as described in section 2.4. PCR products were visualized by 1.2% agarose gel electrophoresis with SYBR Gold nucleic acid gel staining (Invitrogen, USA). The expected PCR fragment sizes were approximately 1126 bp for the first round and 960 bp for the second round of amplification.

2.6 Nucleotide analysis

Following PCR amplification of the ama-1 gene, all positive samples were purified and sequenced by Macrogen (Korea). The resulting nucleotide sequences were analyzed for internal consistency and compared with each other. Additionally, these sequences were compared to previously published *B. bovis* strains deposited in GenBank using the Basic Local Alignment Search Tool (BLAST) available on the National Center for Biotechnology Information (NCBI) website (https://www.ncbi.nlm.nih.gov/).

Table 1 Oligonucleotide primers of *B. bovis* and *B. bigemina*

Target	Sequences 5'-3'	Product size (bp)	References
B.bovis ITS	CGTCCCTGCCCTTTGTA	815	Cao *et al*., 2012
	TATTTTCTTTTCTGCCGCTT		
B.bovis ITS	CACCACCAGTGGAAGCAC	545	
(nestedPCR)	TTGTGCCCCATGGACACT		
B.bigemina ITS	CGTCCCTGCCCTTTGTA	815	Cao *et al*., 2012
	TATTTTCTTTTCTGCCGCTT		
B.bigemina ITS	AGTGGGTCGGGACTCGTC	495	
(nestedPCR)	AGTACCGCCTGCGAGCAG		
Babesia ama-1	TGCCCTTCAGTTGTCCATAGG	1240	Rittipornlertrak *et al*., 2017
(set 1)	CGATATCCAAGTCAGTCGCAG		
Babesia ama-1	CACTCTGTTGGTTCCGCTTTC	1126	Rittipornlertrak *et al*., 2017
(set 2)	GCAGGAGCAATGGCAACACAA		
Babesia ama-1	TACAAGAGCGCTGAGGATGCA	1065	Rittipornlertrak *et al*., 2017
(set 3)	GATTAACAAGCGACCACGATG		
Babesia ama-1	ACCCGGTAAGGGATGCCATTT	960	Rittipornlertrak *et al*., 2017
(set 4)	CACACGGTCAATGAGATGTCT		

2.7 Phylogenetic analysis

Phylogenetic analysis was conducted to investigate the genetic relationships among the *B. bovis* isolates from this study and published strains by using a neighbor-joining method with 1000 bootstrap replicates in MEGA12 software. The *B. bovis* ama-1 sequences from the 33 positive samples were used as templates for the analysis. The *B. bovis* ama-1 isolate from Nakhon Pathom, Thailand (GenBank accession number: KY575957.1), was included as a reference sequence. *B. bigemina* ama-1, *B. divergens* ama-1, and *B. gibsoni* ama-1 sequences were included as outgroups.

3. Results and Discussion

3.1 Prevalence of Babesia infection in Nakhon Si Thammarat

Blood samples were collected from 267 fighting bulls across 13 districts in Nakhon Si Thammarat (Figure1): Nophitam, Phipun, Chawang, Thungyai, Nabon, Changklan, Lansaka, Ronphibun, Chulaphon, Chauat, Huasai, Chianyai, and Pakphanang. Nested PCR analysis identified 33 positive samples from 7 districts, resulting in an overall infection rate of 12.36% (33/267). District-specific infection rates were as follows: Nophitam, 20.0% (4/20); Phipun, 15.0% (3/20); Ronphibun, 31.81% (7/22); Chulaphon, 15.0% (3/20); Chauat, 25.0% (5/20); Huasai, 25.0% (5/20); and Chianyai, 26.09% (6/23) (Table 2).

Nucleotide sequencing and alignment to find the identical sequences confirmed that all positive samples were *B. bovis*; *B. bigemina* was not detected. This finding aligns with previous reports from Chiang Rai, Chiang Mai, and Lampang (Cao *et al*., 2012), Lamphun and Nakhon Pathom (Koonyosying *et al*., 2022), and Nan, Ratchaburi, Nakhon Si Thammarat, Phatthalung, and Surat Thani (Srionrod *et al*., 2022).

The prevalence of tick-borne hemoparasitic infections, particularly Babesia, poses a significant threat to cattle health and the livestock economy. These infections can lead to severe illness, reduced production, and hemolytic anemia, even at low parasitemia levels. Although infected cattle may not exhibit overt clinical signs, low production and post-mortem examination can reveal significant pathological changes (Bock *et al*., 2004; Almazán *et al*., 2022).

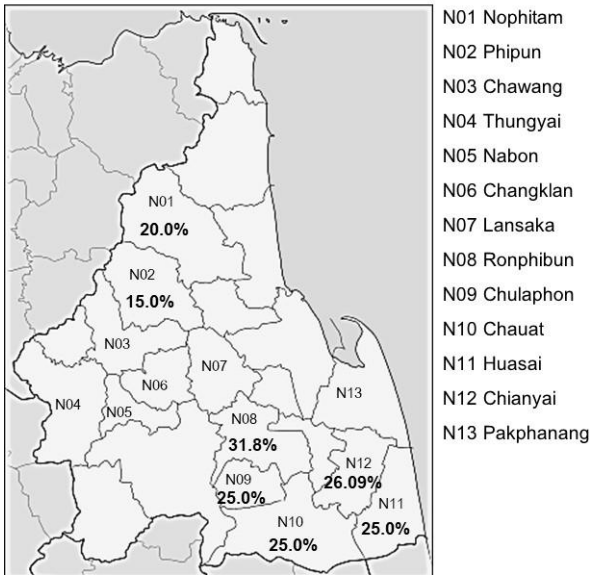

Figure 1 Map of the study area, showing of Nakhon Si Thammarat province. Number for the districts where samples were collected are as follows: N01 Nophitam, N02 Phipun, N03 Chawang, N04 Thungyai, N05 Nabon, N06 Changklan, N07 Lansaka, N08 Ronphibun, N09 Chulaphon, N10 Chauat, N11 Huasai, N12 Chianyai, N13 Pakphanang. The percentage indicated the infection rate in each area.

3.2 Determining B. bovis ama-1 gene

The ama-1 gene was successfully amplified and sequenced from all 33 Babesia-positive samples. Nucleotide BLAST analysis confirmed that all 33 sequences were *B. bovis* ama-1 gene. Within each district group, nucleotide sequences exhibited 100% identity. Across all districts, nucleotide sequence identity ranged from 96.04% to 100.00% when compared to the *B. bovis* isolate from Nakhon Pathom, Thailand (GenBank accession number: KY575957.1) (Table 2). These findings indicate a low genetic distance between the *B. bovis* populations from the seven infected districts and the reference strain (KY575957.1).

3.3 Phylogenetic analysis

Phylogenetic analysis of the ama-1 gene was conducted to elucidate the genetic relationships among *B. bovis* isolates (n=42) using the neighbor-joining method with Maximum Composite Likelihood evolutionary distances (Tamura *et al*., 2004), implemented in MEGA12 (Kumar *et al*., 2024). The resulting phylogenetic tree (Figure 2) revealed a clear genetic differentiation, forming two distinct clades. Clade I, consisting of isolates from the northern districts of Nophitam (N01) and Phipun (N02), demonstrated closer evolutionary relatedness to *B. bovis* isolates from Brazil (FJ588028.1, FJ588027.1, FJ588026.1, FJ588025.1, and FJ588024.1) and other regions of Thailand including isolates from Chiang Rai, Chiang Mai, and Nakhon Pathom (KY575956.1, KY575955.1, and KY575957.1). In contrast, Clade II, comprising isolates from the southern districts of Ronphibun (N08), Chulaphon (N09), Chauat (N10), Huasai (N11), and Chianyai (N12), exhibited close evolutionary distances to *B. bovis* isolates from Israel (KX196263.1, KX196262.1, and AY486101.1) and Sri Lanka (AB787637.1, AB787636.1, AB787635.1, AB787634.1, AB787633.1, and AB787632.1).

This distinct genetic structuring of *B. bovis* populations within Nakhon Si Thammarat has significant implications for disease management. The identification of two geographically associated genetic clades suggests the potential for regional variations in parasite characteristics, such as virulence, vector interactions, and drug susceptibility. For instance, control strategies effective against one clade might not be equally efficacious against the other. Furthermore, this genetic divergence underscores the importance of considering local strain diversity in the development of future interventions, including geographically targeted diagnostic assays and potentially vaccines. The observed genetic links to isolates from Brazil, other parts of Thailand, Israel, and Sri Lanka hint at potential historical or ongoing parasite introductions or shared evolutionary pathways, which could inform broader epidemiological investigations and biosecurity measures. Understanding this genetic heterogeneity is crucial for designing and implementing effective and sustainable control strategies for bovine babesiosis in this economically important fighting bull population.

Table 2 PCR Results, infection rate, and nucleotide sequence similarity of B. bovis ama-1 gene in Nakhon Si Thammarat.

Studied area	No of samples	PCR positive samples	Infection rate	Nucleotide sequences similarity[a]
N01 Nophitam	20	4	20.00%	100.00%
N02 Phipun	20	3	15.00%	100.00%
N03 Chawang	20	0	0.00 %	NA
N04 Thungyai	20	0	0.00 %	NA
N05 Nabon	20	0	0.00 %	NA
N06 Changklan	20	0	0.00 %	NA
N07 Lansaka	22	0	0.00 %	NA
N08 Ronphibun	22	7	31.81%	96.04%
N09 Chulaphon	20	3	15.00%	96.04%
N10 Chauat	20	5	25.00%	96.04%
N11 Huasai	20	5	25.00%	96.04%
N12 Chianyai	23	6	26.09%	96.04%
N13 Pakphanang	20	0	0.00%	NA
Total	267	33	12.36%	NA

[a] Nucleotide sequence similarity analysis was conducted by aligning the sequences to the *B. bovis* ama-1 (Thailand isolate) complete reference sequence KY575957.1

NA = Not available

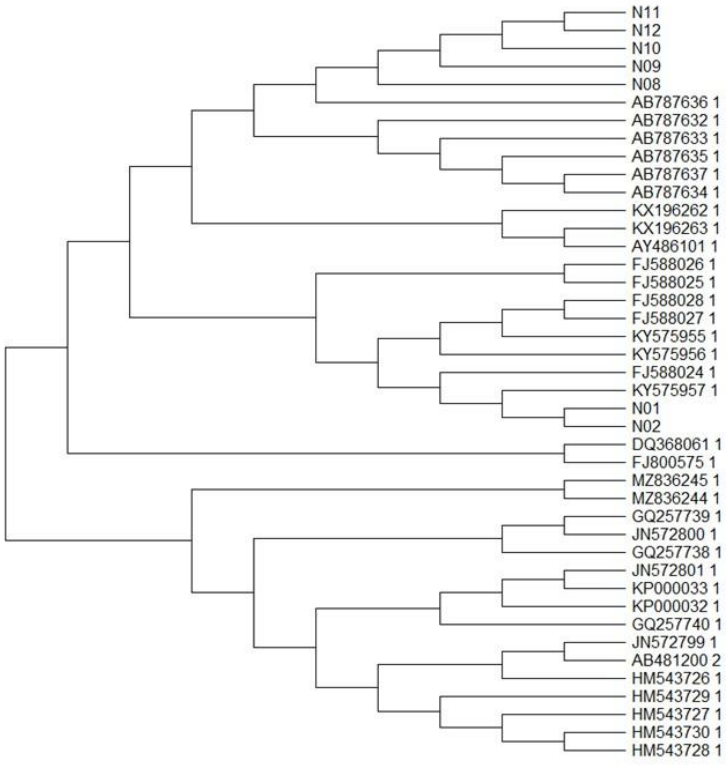

Figure 2 Evolutionary relationships of 42 taxa based on the ama-1 gene.

Phylogenetic analyses were performed using the MEGA12 analysis tools (Kumar *et al*., 2024).

4. Conclusion

In conclusion, this study confirms the presence of *B. bovis* infection in Nakhon Si Thammarat. Nested PCR analysis revealed an overall prevalence of 12.36% (33/267), with the highest prevalence observed in Ronphibun (31.81%, 7/22), followed by Chianyai, Chauat, Huasai, Nophitam, Phipun, and Chulaphon. Phylogenetic analysis of the ama-1 gene sequences from isolates in the northern area of Nakhon Si Thammarat province demonstrated a close genetic relationship with Babesia reported in Brazil and Thailand, while those from the southern area of the province closely resembled those reported in Sri Lanka and Israel. This study lays the groundwork for future research investigating factors contributing to the observed prevalence and the differences between the two phylogenetic clades. This could include vector studies, investigation of clinical manifestations, and assessment of the impact of infection on the bulls' fighting performance.

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