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Research article

Molecular detection of piroplasms in haematophagus flies in the Nakhon Pathom and Kanchanaburi Provinces, Thailand

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Abstract

Theileria spp. and *Babesia* spp are tick-borne protozoan parasites that infect a wide range of domestic and wild animals. In this study, the haematophagus flies (*Stomoxys* and tabanids) from Kanchanaburi and Nakhon Pathom Provinces, Thailand were investigated for the presence of piroplasm DNA. All specimens were morphologically identified. The piroplasms in the insect specimens were first screened by PCR using *Babesia* and *Theleria* 18S rRNA gene and species-specific sequences based on *B. bovis* spherical body protein 2, *B. bigemina* rhoptry-associated protein 1a and *Theileria* spp. Sequencing was used to confirm the identity of all amplicons. In total, 68 blood sucking insects were morphologically identified as *Tabanus* spp. (79.41%), *Stomoxys* spp. (13.24%), *Haematobosca* spp. (5.88%) and *Chrysops* spp. (1.47%). PCR results showed only 2 samples were positive for *Theileria* spp. while all specimens were negative for *Babesia* spp. Based on sequence and phylogenetic analysis, *Theileria* spp. 18S rRNA sequences were classified into *T. ovis* and *T. sinensis* which share high identity and similarity with isolates from other countries. In this study, we describe for the first time the detection of *T. sinensis* in tabanid fly. We therefore recommend further investigations to confirm the biting files as a biological or mechanical vector of theses pathogens, particularly *Tabanus* spp, which should be regarded as a potential vector for transmission of *Theileria* among domestic animals in Thailand.

Keywords: Haematophagus flies, *Theileria* spp., *Babesia* spp., Thailand

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INTRODUCTION

Piroplasmosis caused by *Babesia* and *Theileria* are significant tick-borne protozoan blood parasites that infect a wide range of both domestic and wild animals worldwide (Uilenberg, 1995). *Babesia bovis*, *B. bigemina* are important tick-borne parasites of livestock in tropical and subtropical regions (Bock *et al.,* 2004). Disease manifests as high fever, anorexia and anemia with resultant production losses and mortalities (Bock *et al.,* 2004). Moreover, infection with *B. bovis* cause neurological and respiratory symptoms (Everitt *et al.,* 1986).

Theileria can be grouped into transforming and non-transforming species based on the ability of schizont to invade leukocyte (Sivakumar *et al.,* 2014). The important transforming parasites are *T. annulata* and *T. parva* that are associated with tropical theileriosis and east coast fever, respectively (Mukhebi *et al.,* 1992). *T. orientalis* is responsible for benign or non-transforming theileriosis which is widely distributed in Southeast Asia (Watts, 2016). Although, the parasite is considered to have low pathogenicity it is still able to cause disease which result in weakness, anemia, diarrhea, abortion and poor milk production (Kamau *et al.,* 2011, Izzo *et al.,* 2010).

Generally, *Babesia* and *Theileria* parasites are transmitted by Ixodid ticks (Mehlhorn and Schein, 1985). However, other biting arthropods including mosquitoes, tabanid flies and lice can serve as mechanical vectors for *Theileria* spp. and may be responsible for disease transmission in livestock (Fujisaki *et al.,* 1993; Baldacchino *et al.,* 2013; Hammer *et al.,* 2016). Distribution and abundance of haematophagus flies (*Stomoxys* and tabanids) in Thailand have been reported previously (Tumrasvin, 1989; Changbunjong *et al.,* 2018). Most molecular studies on the biology of the biting flies are focused on identification and genetic variation. However, information on the blood protozoan parasites in the insects have not yet been elucidated. Therefore, our current work is investigating the presence of piroplasm in haematophagus flies that were found in animal farms using polymerase chain reaction (PCR) technique. Phylogenetic relationship among the isolates identified in this study and those isolated from different countries were compared.

MATERIALS and METHODS

Study site and sample collection

A cross sectional study was employed to collect flies sample between June and July, 2015. This study was carried out in three different locations in Kanchanaburi and Nakhon Pathom Provinces. All locations were situated near beef cattle farms.

In each study location, fly samples were collected by using Nzi trap. Flies were collected and all samples were labeled separately by location and date. Those samples were placed into a cooler containing cool ice packs and transported to the laboratory. Collected flies were identified based on morphology of the flies as previously described (Zumpt, 1973; Tumrasvin and Shinonaga, 1978). Only female flies were included in this study. The samples were preserved in 95% ethanol and stored at -20°C until further use.

DNA extraction

Each fly was washed three times in 70% ethanol and once in sterile water and then dried. Individual fly bodies were homogenized with a sterile pellet mixer in 1.5 ml tubes. The DNA was then extracted after the flies were crushed using standard proteinase K digestion, phenol/chloroform extraction and ethanol precipitation (Hunt, 1997). The pellets were resuspended in 100 µl of double distilled water. The DNA concentration was measured using NanoDrop™ 2000/2000c spectrophotometers (ThermoFisher Scientific, USA) and then stored at -30°C until processed.

PCR detection of *Babesia* and *Theileria*-specific DNA

Sixty-eight specimens detected for the presence of blood protozoan parasites using PCR assay with genus-specific primers followed by species-specific primers (Table 1). All samples were first screened for the presence of *Babesia* and *Theleria* species by targeting the hypervariable V4 region of the 18S rRNA gene (Georges *et al.,* 2001). Single-step PCR assays were performed in a final volume of 20 µl, containing 100 ng of DNA sample, 0.25 mM of dNTPs, 1X of PCR buffer, 1 pmol of each primer (Sigma Aldrich, Japan) and 0.1 U of Taq-polymerase (Ex-taq DNA polymerase, Takara, Japan).

Positive samples for the piroplasm were then detected for *B. bovis*, *B. bigemina* and *Theileria* species. Previously described specific PCR primers for *B. bovis* spherical body protein 2, *B. bigemina* rhoptry-associated protein-1a and *Theileria* 18S rRNA were used as markers in this study (AbouLaila et al., 2010; Terkawi et al., 2011; Cao et al., 2013). Nested PCR assays were performed in 20 µl of reaction mixture containing 100 ng of extracted DNA, 0.25 mM of dNTPs, 1X of PCR buffer, 1 pmol of each primer (Sigma-Aldrich, Japan), 1 U of Taq-polymerase (Ex-Tag DNA polymerase, Takara, Japan) and topped up with distilled water to the final volume. For the inner primer reaction, 2 µl of the outer PCR products were added into the reaction mixture. Positive and negative quality controls were include in each experiment. The PCR product were subjected to electrophoresis on 1.5% agarose gel in 1X TAE buffer, stained with ethidium bromide, and visualized under UV light.

Identification of *Babesia* and *Theileria* DNAs

PCR products of the correct size were identified by sequencing. Amplification products were purified from agarose gel by using Nucleospin® Extract II (Macherey Nagel, Germany). The fragments were cloned into a pGEM-T Easy Vector (Promega, USA). After transformation, two recombinant clones were selected for sequencing using a Dye Terminator Cycle Sequencing Kit (Applied Biosystems, USA) and the DNA sequences were determined using ABI PRISM 3100 genetic analyzer (Applied Biosystems, USA). The sequence analysis was performed using Bioedit version 7.2.5 (Tom Hall Ibis Biosciences, USA). The nucleotide sequence identities and similarities of obtained sequence were analyzed by BLASTn search in GenBank and percent identities between nucleotide sequences were compared by Pairwise distance based on p-distance method using MEGA version 6.0 program (Tamura *et al.,* 2013).

Nucleotide sequence accession numbers

The nucleotide sequences obtained in this study were registered in the GenBank under the accession numbers MH156035 and MH156036.

Table 1 List of primers used for PCR assays.

Target	Primer sequences (5'-3')	Fragment (bp)	Reference
Theileria and *Babesia* species18S rRNA gene (PCR)	For-CTAAGAATTTCACCTCTGACAGT Rev-GACACAGGGAGGTAGTGACAAG	403	Georges *et al.*, 2001
B. bovis SBP2 gene (nPCR)	For [a]-CTGGAAGTGGATCTCATGCAACC For [b]-GAATCTAGGCATATAAAGCAT Rev-TCACGAGCACTCTACGGCTTTGCAG ATCCCCTCCTAAGGTTGGCTAC	1,236 580	AbouLaila *et al.*, 2010
B. bigemina RAP-1a gene (nPCR)	For [a]-GAGTCTGCCAAATCCTTAC For [b] AGCTTGCTTTCACAACTCGCC Rev-TCCTCTACAGCTGCTTCG TTGGTGCTTTGACCGACGACAT	879 412	Terkawi *et al.*, 2011
Theileria 18S rRNA gene (nPCR)	[a]GAAACGGCTACCACATCT [b]TTAAACCTCTTCCAGAGT Rev-AGTTTCCCCGTGTTGAGT TCAGCCTTGCGACCATAC	778 581	Cao *et al.*, 2013

nPCR = Nested PCR, [a] = Outer primers, [b] = Inner primers

RESULTS

Species identification

A total 68 blood sucking samples were identified to 5 genera and 10 species. The most abundant species in this study were *Tabanus oxybeles* (45.59%) followed by *Tabanus megalops* (22.06%), *Stomoxys calcitrans* (8.82%), *Tabanus rubidus* (7.35%), and *Chrysops dispar* (2.94%). Number and percentage of blood sucking flies are described in Table 2.

Detection of *Babesia* and *Theileria* spp.

Two samples from the 68 specimens produced an amplicon of a size corresponding with the 18S rRNA of piroplasms. The 2 positive samples were further tested for *B. bovis*, *B. begemina* and *Theileria* spp. The PCR results revealed that both samples were positive with Theileria 18S rRNA while *B. bovis* and *B. bigemina* were not detected (Table 2). Species of *Theileria* spp. were identified by sequencing and compared with GenBank database. The sequence analysis showed that one partial sequence (MH156035) shared high similarity with *T. ovis* isolates and shared 98.80-98.97% identity with published sequences (Table 3). The other partial sequence (MH156036) showed high similarity with *Theileria* spp and *T. sinensis* and shared 99.48-99.83% identity value with database sequences (Table 4).

Table 2 The results of PCR method showing positive results for *Theileria* spp. in haematophagus flies

Province	District	Species	Total number of collected samples (%)	Number of positive samples (%)
Nakhon Pathom	Kumpangsan	*Tabanus megalops*	14 (20.59)	2 (14.28)
		Tabanus rubidus	2 (2.94)	0
		Stomoxys calcitrans	2 (2.94)	0
		Chrysops dispar	2 (2.94)	0
Kanchanaburi	Thongphaphum	*Tabanus oxybeles*	31 (45.59)	0
	Loakwan	*Tabanus rubidus*	3 (4.41)	0
		Tabanus megalops	1 (1.47)	0
		Tabanus rhinargus	1 (1.47)	0
		Tabanus rubicundus	1 (1.47)	0
		Tabanus tamthaiorum	1 (1.47)	0
		Stomoxys pullus	2 (2.94)	0
		Stomoxys calcitrans	4 (5.88)	0
		Stomoxys sp.	1 (1.47)	0
		Haematobosca sanguinolenta	3 (4.41)	0

Table 3 Percent identity of *T. ovis* from GenBank database and Thai *Theileria* spp. by Clustal W.

Accession number	1	2	3	4	5	6	7	8
1. MH156035, *Theileria* spp., Thailand								
2. JQ737135, *T. ovis*, Iran	98.97							
3. KR094869, *T. ovis*, Iraq	98.97	100.00						
4. KM924444, *T. ovis*, Tunisia	98.97	100.00	100.00					
5. FJ603460, *T. ovis*, China	98.97	100.00	100.00	100.00				
6. EU622911, *T. ovis*, France	98.97	100.00	100.00	100.00	100.00			
7. AY533144, *T. ovis*, Spain	98.97	100.00	100.00	100.00	100.00	100.00		
8. AY260172, *T. ovis*, Turkey	98.97	100.00	100.00	100.00	100.00	100.00	100.00	
9.AY260171, *T. ovis*, Germany	98.80	99.83	99.83	99.83	99.83	99.83	99.83	99.83

Table 4 Percent identity of *T. sinensis* from GenBank database and Thai *Theileria* spp. by Clustal W.

Accession number	1	2	3	4
1. MH156036, *Theileria* spp. Thailand				
2.AB000270, *Theileria* spp.Thung Song, Thailand	99.83			
3. KX115427, *T. sinensis*, China	99.48	99.65		
4. HM538203, *T. sinensis*, China	99.48	99.65	100.00	
5. EU274472, *T. sinensis*, China	99.48	99.65	100.00	100.00

A phylogenetic tree of *T. ovis* and *Theileria* spp. was constructed from *Theileria* spp. 18S rRNA gene sequences of our amplicons and those available in GenBank. As shown in Figure 1, the isolate from Kampangsan (MH156035) formed a cluster with the *T. ovis* gene sequences previously isolated from Turkey, Iraq, Iran, China and France. The sequence MH156036 was clustered into the same clade together with *Theileria* spp. from Thailand and *T. sinensis* from China.

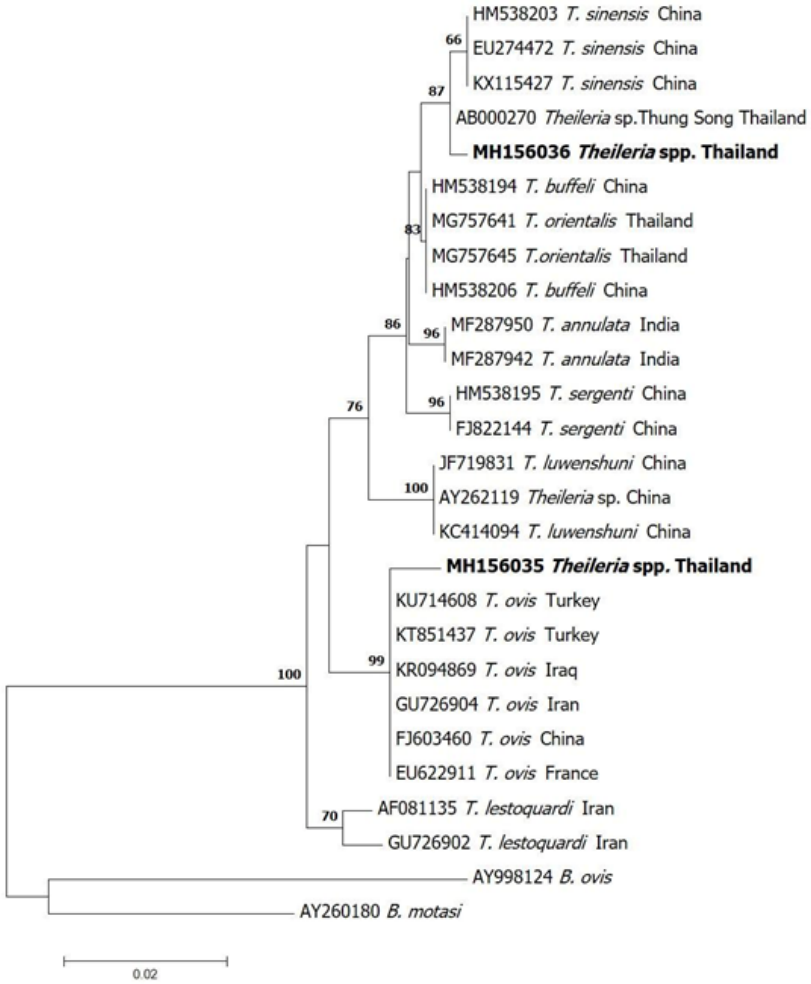

Figure 1 Phylogenetic analysis of *Theileria* spp. 18S rRNA gene sequences in *Tabanus* spp. samples (boldface letters) and previously registered sequences in the GenBank database. Phylogenetic tree were constructed based on neighbor-joining method with 1,000 replicates. GenBank accession number are shown. Bootstrap values are provided at the beginning of each branch. The 18S rRNA gene of *B. ovis* (AY998124) and *B. motasi* (AY260180) were used as outgroups.

DISCUSSION

A previous molecular detection of *Theileria* and *Babesia* in tabanids and Stomoxyini flies was carried out in Khao Yai National Park, Thailand (Changbunjong et al., 2016). However, the presence of the above parasites in biting flies in farm livestock has not yet been reported. In this study, we collected 68 insect samples from three animal farms and 10 species were identified (Table 2). The distribution of those species has been reported throughout of Thailand (Tumrasvin, 1989; Changbunjong *et al.,* 2018). Among them, *Tabanus* spp. were observed with the greatest numbers in the study area. Thus, our result was similar with the finding of Changbunjong *et al* (2018) who reported *Tabanus* spp. was the most abundant occurring fly on beef cattle and buffalo farms. The number of *Stomoxys* flies in this study was lower than previous observation on animal farms (Phasuk *et al.,* 2013). The authors attributed this to the effectiveness of trap type. Tunnakundacha *et al* (2017) reported that the Nzi trap caught significantly higher numbers of *Tabanus* spp. than the other traps. In addition, factors such as the season and temperature have been shown to determine the abundance of *Tabanus* and *Stomoxys* flies in Thailand (Phasuk *et al.,* 2010).

The sequence analysis of hypervariable V4 region of the 18S rRNA gene in this study revealed that 2 DNA samples of *Tabanus* spp. were positive for *Theileria* spp. This was consistent with Changbunjong *et al* (2016) reporting that *Theileria* spp. and *Babesia* spp. were detected in blood sucking insect samples. However, we could not detect *Babesia* spp. positive samples from the surveyed areas probably due to the low number of samples. Therefore, a large-scale study with an increased number of samples from different areas needs to be undertaken.

Generally, *Theileria* spp. are transmitted by ixodid ticks of the genera *Amblyomma*, *Haemaphysalis*, *Hyalomma* and *Rhipicephalus*. Previous study of Hadi and Al-Amery (2011) reported *Theileria* and *Babesia* parasites detected in midgut of *S. calcitrans* by Giemsa staining. Therefore, the presence of the parasite DNA probably due to host blood meal consumed from infected animals. To confirm the mechanical transmission, the DNA extraction from specific organs such as salivary gland, mouthparts or digestive tracts should be examined. Recently, previous studies have shown the evidence for mechanical transmission by other biting arthropods including mosquitoes, tabanid flies, and lice (Hammer et al., 2016; Fernández de Marco *et al.,* 2016). In this study, we detected *Theileria* spp. in several samples but it is unclear that tabanid flies can serve as mechanical vector for *Theileria* spp. Further studies on the modes of transmission of the pathogen in blood sucking insects are required to clarify our finding.

Different *Theileria* spp., so far, including *T. luwenshuni*, *T. orientalis*, *T. separate*, *T. ovis*, *T. lestoquardi*, *T. uilenbergi*, *T. recondite, T. annulata* and *T. parva* have been reported in livestock animals (Mans *et al.,* 2015). The 18S small subunit ribosomal RNA (18S rRNA) gene is widely used in the detection of *Theileria* spp. Furthermore, the 18S rRNA gene has been successfully applied to distinguish previously unknown *Theileria* spp. (Cao *et al.,* 2013). Hence, we used *Theileria* 18S rRNA gene for the detection and genetic characterization of the parasite.

T. ovis is responsible for ovine theileriosis and cause benign infections in small ruminants worldwide (Mans *et al.,* 2015). In Thailand, there is some published information of *Theileria* infection in goat from Sa Kaeo Province reported by Kaewhom and Thitasan (2017). Furthermore, the species has not yet been identified. Here, we report the first identification of *T. ovis* isolated from an insect specimen. The sequence of *T. ovis* in this study showed 98% identity to *T. ovis* that was detected from goat and sheep in Turkey, Iraq, Tran, China and France. Recently, *T. ovis* was observed in deer keds (Lipoptena fortisetosa) from South Korea and also the sequence showed 100% identity to the parasite obtained from Chinese water deer (Lee *et al.,* 2016). According to these findings, the high similarity between the obtained sequence and previous published sequence indicates this fly could potentially play a significant role in the parasite transmission in animals.

The *Theileria* 18S rRNA gene sequence in this study shared a high identity with *T. sinensis* that was isolated from cattle in China. Moreover, the obtained sequence also formed one cluster with the other *T. sinensis* sequences in phylogenetic analysis. According to the findings, the sequence MH156036 that was originally submitted simply as *Theileria* sp., we now propose that sequence is *T. sinensis*. This parasite is a new species that was first isolated from cattle in China (Bai *et al.,* 2002) and classified as benign Theileria species (Liu *et al.,* 2012). In Thailand, *T. orientalis* has long been considered a benign parasite and widely distributed in dairy and beef cattle (Jirapattharasate *et al.,* 2017; Altangeral et al., 2011). Here, we report the first detection of *T. sinensis* in the tabanid fly from Thailand. This finding could be attributed to the circulation of parasite infection on animal farms, therefore, further studies on *T. sinensis* prevalence in cattle and arthropod vectors in Thailand should be performed.

CONCLUSION

In conclusion, *T. ovis* and *T. sinensis* were detected in tabanid flies. However, further investigations are required to confirm the biting files as a biological or mechanical vector of theses pathogens. We therefore recommend further studies on the prevalence of these 2 parasites in livestock animals. In addition, natural vectors in animal farms or an environment should be investigated and examined the parasites. These information could be improved the understanding of the blood parasites circulating in animal herds in Thailand

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CONFLICT OF INTEREST

The authors declared that there is no conflict of interest.

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