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RESEARCH ARTICLE

Molecular screening of *Babesia*, *Hepatozoon,* and *Theileria* (Apicomplexa: Piroplasmida) in ticks (Acari: Ixodidae) infesting farm ruminants in Peninsular Malaysia

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ABSTRACT

Ticks are obligate hematophagous arachnids that feed on both humans and animals. Despite the extensive research on detection of bacteria in ixodid ticks in Malaysia, there remains limited knowledge about the detection of protozoa in these ectoparasites, especially in those that feed on farm ruminants. In this study, 1,241 ticks belonging to four species (*Rhipicephalus microplus, R. haemaphysaloides, Haemaphysalis bispinosa,* and *H. wellingtoni*) were collected from 674 farm ruminants across Peninsular Malaysia. The ticks were pooled and subjected to DNA extraction, followed by protozoal screening using 18S rRNA gene polymerase chain reaction (PCR). Of the 130 tick pools tested, 15 were positive for *Babesia* (11.54%) and ten for *Theileria* (7.69%). No *Hepatozoon* protozoa were detected. All positive pools consisted solely of *R. microplus* ticks, with no protozoa found in the other three tick species. BLAST analyses revealed that the *Babesia* sequences were identical to *Babesia bigemina*, while the *Theileria* sequences closely resembled *Theileria orientalis* and *Theileria sinensis*. This paper presents the first nationwide screening of *Babesia, Hepatozoon,* and *Theileria* in ticks infesting farm ruminants from Peninsular Malaysia.

Keywords: Piroplasmids; tick-borne diseases; veterinary parasitology; ectoparasites; Malaysia.

INTRODUCTION

Ticks are obligate blood-feeding acari that parasitize a diverse range of hosts across various classes and orders (Guglielmone *et al*., 2014; Kazim *et al*., 2022). They are globally ranked as the second most medically important arthropods after mosquitoes, primarily due to their ability to harbor and transmit a variety of pathogens to humans (Service, 2012; Sonenshine & Roe, 2013). Ticks are known vectors of protozoan infections, such as babesiosis, caused by *Babesia* species, and theileriosis, caused by *Theileria* species (Araya-Anchetta *et al*., 2015). Additionally, ticks act as both definitive hosts and vectors for *Hepatozoon*, a primary causative agent of hepatozoonosis (Baneth *et al*., 2007).

Babesia is the second most prevalent blood parasite in mammals and birds, with a global distribution (Schnittger *et al*., 2012; Svensson *et al*., 2019). The protozoa are commonly transmitted by *Rhipicephalus* and *Ixodes* ticks. In cattle, *Babesia bigemina* is a significant species that can lead to considerable economic losses due to symptoms like anemia, fever, jaundice, reduced productivity, weight loss, and, in severe cases, death (Bock *et al*., 2004; Jonsson *et al*., 2008; Martins *et al*., 2008; Tembue *et al*., 2011; Pupin *et al*., 2019). In Malaysia, several studies have documented various *Babesia* species isolated from domestic animals, livestock, and wildlife, including *Babesia bigemina*, *Babesia bovis*, *Babesia canis*, *Babesia gibsoni*, and *Babesia vogeli* (Chandrawathani *et al*., 1994; Mokhtar *et al*., 2013; Mohammed *et al*., 2017; Prakash *et al*., 2018a; Ola-Fadunsin *et al*., 2021). A unique *Babesia* species was also detected from the Bornean sun bear (*Helarctos malayanus euryspilus*) at the Bornean Sun Bear Conservation Centre in Sabah (Chua *et al*., 2022). Furthermore, Lau *et al*. (2022) reported a *Babesia* protozoan closely related to a marsupial-associated species in Sarawak, which was detected from an alleged male *Haemaphysalis shimoga* tick infesting a rodent in an oil palm plantation. The authors identified the tick species using the 16S rRNA gene sequence but did not provide morphological evidence to support the molecular findings.

Theileria infects blood and lymphatic systems of various domestic and wild ruminants (Remesar *et al*., 2021). Notable species like *Theileria parva* and *Theileria annulata* are highly pathogenic and cause diseases such as East Coast fever and tropical theileriosis, which are often fatal in cattle (Mans *et al*., 2015). Other species, such as *Theileria orientalis* and *Theileria sinensis*, are less pathogenic but can still negatively impact cattle health (Chaisi *et al*., 2014; Perera

et al., 2014). Theileriosis is characterized by symptoms such as fever, lymphadenopathy, anaemia, and, in severe cases, death. In Malaysia, several *Theileria* species have been reported in ticks and farm ruminants. Kho *et al*. (2017) identified *Theileria buffeli*, *Theileria sergenti*, and *T. sinensis* in cattle, as well as *Theileria luwenshuni* in infected sheep. Additionally, *T. buffeli* and an undescribed *Theileria* species were detected in *Haemaphysalis bispinosa* and *R. microplus*, respectively. Lim *et al*. (2019) also detected *T. luwenshuni* from *H. bispinosa* ticks infesting goats on a private farm in Perak.

Hepatozoon, on the other hand, infects tissues of vertebrates, including mammals, reptiles, and birds (Hodžić *et al*., 2018; Keel *et al*., 2018). Unlike many other tick-borne protozoa, *Hepatozoon* is transmitted when the host ingests an infected tick or other arthropod vector, rather than through a tick bite (Smith, 1996). Symptoms of *Hepatozoon* infections vary depending on the species and host but can include fever, lethargy, anaemia, and weight loss (Allen, 2022). In Malaysia, the knowledge of tick-borne *Hepatozoon* is limited, with most research focusing on companion animals such as dogs (Rajamanickam *et al*., 1985; Mohammed *et al*., 2017; Low *et al*., 2018; Prakash *et al*., 2018b; Premaalatha *et al*., 2018). Perison *et al*. (2022) reported that two out of 40 rat blood samples (15.4%) tested positive for *Hepatozoon ophisauri* in southern Sarawak. Recent studies have also reported occurrences of *Hepatozoon* species in wild and farm ruminants from other parts of the world (Tila *et al*., 2023; Uiterwijk *et al*., 2023). Due to the host specificity of some *Hepatozoon* species (e.g., *H. canis* in dogs) (Duszynski *et al*., 2018), no studies have yet focused on detecting this protozoan in Malaysian farm ruminants.

Currently, information on *Babesia*, *Hepatozoon*, and *Theileria* in ticks from ruminants is limited in Malaysia. Therefore, this study presents a survey of these protozoa in ticks collected from selected ruminant farms in Peninsular Malaysia.

MATERIALS AND METHODS

Animal ethics approval

This study adhered to the guidelines for the care and use of animals as approved by the Committee of Animal Research and Ethics at Universiti Teknologi MARA (UiTM CARE), with ethical approval number UiTM CARE: 378/2022. Additionally, it received approval from the Research Project Evaluation Committee of the Malaysian Department of Veterinary Services, under reference number JPV. BPI.600-1/7/1 (2021-14).

Study sites, tick collection, and morphological identification

Ruminant farms in Peninsular Malaysia were visited over the course of one year, from October 2020 to November 2021. Farms were randomly selected across four regions of the peninsula: the central region (Selangor and Kuala Lumpur), the southern region (Melaka and Johor), the eastern region (Pahang), and the northern region (Perak) (Figure 1). Both large ruminants (i.e., cattle and buffaloes) and small ruminants (i.e., goats and sheep) were examined for tick infestations. Ticks were extracted using fine tweezers and preserved in tubes containing 90% ethanol. These samples were transported to the parasitology laboratory at the Institute of Medical Molecular Biotechnology (IMMB), UiTM Sungai Buloh Campus, for morphological identification. Using an Olympus SZX7 Zoom stereo microscope (Olympus Europa SE & Co. KG, Germany), the ticks were identified based on taxonomic keys from Anastos (1950), Kohls (1957), Trapido *et al*. (1964), Tanskul & Inlao (1989), and Walker *et al*. (2000).

DNA extraction and molecular analyses of tick-borne protozoa

Ten ticks were pooled for DNA extraction, while fully engorged females and nymphs were processed individually. After morphological identification, different species were kept separate during the extraction process. The ticks were initially washed with distilled water and dried using KIMWIPES delicate wipers (Kimberly-Clark Professional, CA), before being transferred to 1.5 mL microcentrifuge tubes and dissected with sterile surgical scissors (S.S. Pakistan, Pakistan). DNA extraction was performed using the DNEasy® Blood and Tissue Kit (QIAGEN©, California), following the manufacturer's protocol. The eluted DNA was subjected to conventional PCR using three sets of 18S rRNA primers specific for *Babesia*, *Theileria*, and *Hepatozoon* DNA (Table 1).

PCR amplification was conducted in a 25 µL reaction volume, containing 12.5 µL of GoTaq® Green master mix (Promega, Wisconsin), 4 µL of DNA template, 1 µL of each forward and reverse primer, and 6.5 µL of nuclease-free water (QIAGEN©, California). Amplification followed cycling conditions outlined in reference studies (Table 1) using a Biometra TAdvanced Twin 48 thermal cycler (Analytik Jena GmbH+Co. KG, Germany). A negative control was included in each PCR run to monitor for contamination. Amplicons were analyzed by gel electrophoresis using a 1.7% agarose gel stained with 1.5 µL FloroSafe DNA stain (1st BASE, Singapore) and visualized under UV light. PCR products were sent to an external company (Apical Scientific Sdn. Bhd., Selangor) for DNA sequencing. Sequences were aligned and trimmed using BioEdit software (Hall, 1999), then analyzed for similarities using BLAST on the NCBI website.

Phylogenetic tree construction

Phylogenetic trees were constructed for *Babesia* and *Theileria* using 18S rRNA gene sequences. Sequences closely related to those identified through BLAST searches and other relevant sequences of valid species were incorporated into tree construction. The Neighbor-Joining method in MEGA11 (Tamura *et al*., 2021) with 1000 bootstrap replicates was used to infer phylogenetic relationships. *Hepatozoon canis* (KX880503) and *Toxoplasma gondii* (L24381) were used as outgroups for the analysis of *Babesia* and *Theileria*, respectively.

RESULTS

A total of 1,241 ticks were collected from 674 farm ruminants in Peninsular Malaysia (Table 2) – *Rhipicephalus microplus* (1,229/1,241 ticks; 99.03% prevalence), *Rhipicephalus haemaphysaloides* (6/1,241 ticks; 0.48% prevalence), *Haemaphysalis bispinosa* (5/1,241 ticks; 0.40% prevalence), and *Haemaphysalis wellingtoni* (1/1,241 ticks; 0.08% prevalence). Table 3 shows the prevalence of ticks according to species and region. For the molecular screening of *Babesia*, *Theileria*, and *Hepatozoon*, 130 tick pools were prepared (Table 4). Among these, 15 pools (11.54%) were positive for *Babesia*, and 10 pools (7.69%) were positive for *Theileria*. The southern region had the highest number of *Babesia*-positive pools, accounting for 60.0% (9/15 pools), followed by the eastern region with 33.33% (5/15 pools), and the central region with 6.67% (1/15 pools). Notably, no *Babesia* protozoa were detected in the northern region. In contrast, the central region showed the highest number of *Theileria*-positive pools at 40.0% (4/10 pools), followed by the eastern region at 30.0% (3/10 pools), the southern region at 20.0% (2/10 pools), and the northern region with 10.0% (1/10 pools). No co-infection of *Babesia* and *Theileria* was detected throughout the study. All positive pools were from *R. microplus* ticks, while no protozoa were detected in *R. haemaphysaloides*, *H. bispinosa*, and *H. wellingtoni*. Similarly, no *Hepatozoon* protozoa were detected in any of the pools.

All 15 *Babesia* sequences showed high similarity (99.75–100%) to *Babesia bigemina*. Six of the ten *Theileria* sequences (B11, B32, B48, B81, B114, and BS9) were closely similar or identical to *Theileria orientalis* (99.01–100%). Two additional sequences (B6 and B17) showed close similarity (99.22–99.77%) to *Theileria annulata* but clustered with *T. orientalis* in the phylogenetic tree analyses. Further investigation of the BLAST hits revealed that these sequences were also similar to *T. orientalis*, with the same percentage similarities

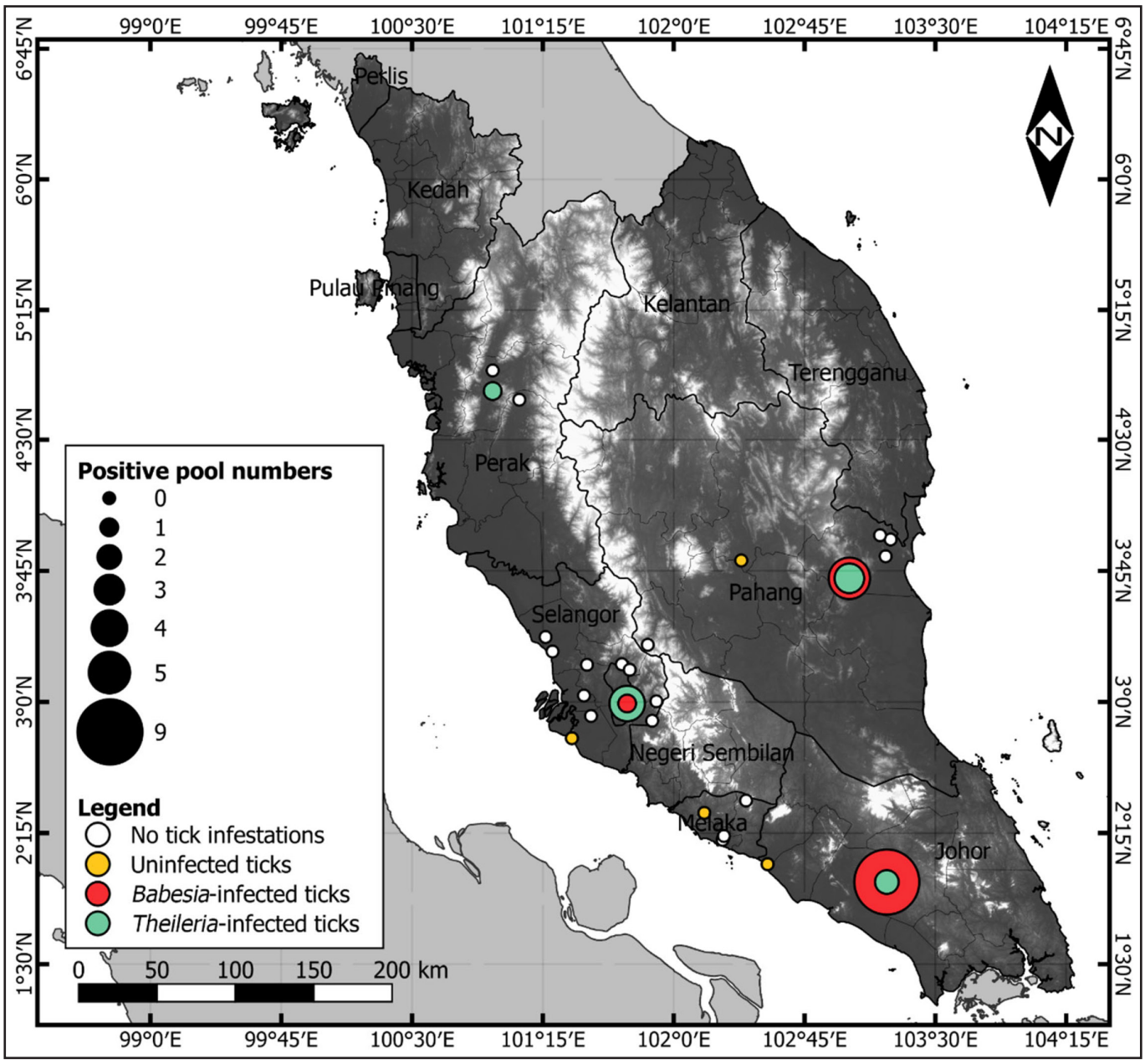

Figure 1. Map of the Peninsular Malaysia, illustrating the visited farms and the geographical distribution of *Babesia* and *Theileria*. Map shapefiles of Peninsular Malaysia and other neighbouring countries were downloaded from https://www.gadm.org. The digital elevation model (DEM) data for Peninsular Malaysia was obtained from the United States Geological Survey (USGS) website.

Table 1. Primers used to screen the protozoa in this study

Target gene	Primer name	Primer sequence	Base pair size (bp)	Annealing temperature, T_a (°C)	Reference
Babesia 18S rRNA	BAB143-167 BAB694-667	F: CCG TGC TAA TTG TAG GGC TAA TAC R: GCT TGA AAC ACT CTA RTT TTC TCA AAG	~551	58	Almeida *et al*. (2012)
Hepatozoon 18S rRNA	HepF HepR	F: ATA CAT GAG CAA AAT CTC AAC R: CTT ATT ATT CCA TGC TGC AG	~656	57	Inokuma *et al*. (2002)
Theileria 18S rRNA	Tbs-S Tbs-A	F: CAC AGG GAG GTA GTG ACA AG R: CTA AGA ATT TCA CCT CTG ACA G	~450	55	Ghaemi *et al*. (2012)

as with *T. annulata*. The last two sequences (B5 and B8) were closely related or identical to *Theileria sinensis*. Sequence B5 was 99.53% similar to *Theileria* sp. isolate Thung Song (AB000270) and 99.30% similar to *Theileria sinensis* isolate T64 (MT271911), while sequence B8 was 100% identical to *Theileria sinensis* isolate CDA9102 (MZ314843). Figure 2 illustrates the clustering of the *Babesia* sequences in this study with *Babesia bigemina* (Figure 2A) and the *Theileria* sequences with *T. orientalis* and *T. sinensis* (Figure 2B).

All protozoa sequences were submitted to the GenBank database with their respective accession codes: *Babesia* (PP267074, PP318198–PP318211) and *Theileria* (PP267992–PP267995, PP317917–PP317921, PP488287).

Table 2. Prevalence of infested large and small ruminants, and the number of ticks, according to region

Region	Animal host (*n*)	Infected animal host (%)	Number of ticks collected
Central	Large ruminants (92)	40 (43.48)	149
	Small ruminants (38)	0 (0)	–
Southern	Large ruminants (94)	61 (55.96)	197
	Small ruminants (75)	0 (0)	–
Eastern	Large ruminants (129)	111 (86.04)	864
	Small ruminants (101)	0 (0)	–
Northern	Large ruminants (107)	26 (24.30)	31
	Small ruminants (38)	0 (0)	–
Total	674	238 (35.31)	1,241

DISCUSSION

The *Babesia* samples obtained in this study were identified as *Babesia bigemina*, a significant pathogen affecting cattle. This pathogen was detected in all regions of Peninsular Malaysia except for the northern region, which might be due to the low number of tick specimens collected from that area.

Nur Mahiza (2010) reported a very low prevalence of *B. bigemina* (0.1%), whereas Rahman *et al*. (2010) found a prevalence of 16.0% for *B. bigemina* and 17.0% for *B. bovis* in Peninsular Malaysia. In contrast, Ola-Fadunsin *et al*. (2021) reported a much higher prevalence of 30.5% for *B. bigemina* based on serological tests using cattle blood samples. These discrepancies in prevalence could be due to differences in diagnostic methods. For example, Nur Mahiza (2010) used conventional microscopy to detect *B. bigemina*, while Rahman *et al*. (2010) utilized serological techniques, which may be more sensitive. Additionally, the type of samples used for screening could also explain the differences in prevalence. Ola-Fadunsin *et al*. (2021) used cattle blood samples, and it is likely that infected cattle with active parasitemia would show a higher prevalence of the protozoan. Furthermore, McCoy *et al*. (2014) suggested that the low number of endoparasites in ticks could result from recent tick infestations on cattle, which might provide insufficient time for the protozoa to establish in the tick midgut during blood feeding. Interestingly, no *B. bovis* was detected in any of the 130 tick pools, and the reason for its absence remains unknown.

Babesia bigemina is an important parasite that negatively affects cattle health, causing signs such as fever, hemoglobinuria, and anemia (World Organization for Animal Health, 2021). No abnormal signs were observed in the cattle during tick collection, which may

Table 3. Tick prevalence according to species and geographical region in Peninsular Malaysia

Geographical region	Tick species				Total	Prevalence (%)
	Haemaphysalis bispinosa	*Haemaphysalis wellingtoni*	*Rhipicephalus haemaphysaloides*	*Rhipicephalus microplus*		
Central	0	0	5	144	149	12.00
Southern	2	0	0	195	197	15.87
Eastern	2	1	1	860	864	69.62
Northern	1	0	0	30	31	2.50
Total	5	1	6	1,229	1,241	100.00
Prevalence (%)	0.40	0.08	0.48	99.03	100.00	

Table 4. Allocation of tick pools for DNA extraction and PCR, and the number of pools positive for *Babesia* and *Theileria*

Region	Farm	Tick species (n)	Number of ticks	Total pools	Total positive for *Babesia*/*Theileria*
Central	4	*Rhipicephalus haemaphysaloides*	5	1	0/0
		Rhipicephalus microplus	14	2	0/0
	10	*Rhipicephalus microplus*	130	13	1/4
Southern	6	*Haemaphysalis bispinosa*	2	1	0/0
		Rhipicephalus microplus	5	1	0/0
	7	*Rhipicephalus microplus*	190	19	9/2
Eastern	5	*Haemaphysalis bispinosa*	2	1	0/0
		Haemaphysalis wellingtoni	1	1	0/0
		Rhipicephalus microplus	730	73	5/3
	6	*Rhipicephalus haemaphysaloides*	1	1	0/0
		Rhipicephalus microplus	130	13	0/0
Northern	2	*Haemaphysalis bispinosa*	1	1	0/0
		Rhipicephalus microplus	30	3	0/1
Total			1,241	130	15/10

indicate that the cattle act as reservoirs for *B. bigemina* (Iseki *et al*., 2010).

In contrast to *Babesia*, *Theileria* was detected in all four regions of Peninsular Malaysia, including the northern region. Although the overall prevalence of *Theileria* was lower than that of *Babesia* (7.69% vs. 11.54%), *Theileria* showed a wider geographical distribution. Kho *et al*. (2017) reported that 10.3% of cattle ticks (seven out of 68) tested positive for *Theileria*. Sequence analysis of the 18S rRNA gene of *Theileria* spp. found in a *R. microplus* tick revealed 99.7% similarity to *Theileria* sp. type C (U97051), while a sequence from a *H. bispinosa* tick demonstrated 99.7% similarity to *Theileria buffeli* Warwick-Australia (AB000272). Apart from that, Rohaya *et al*. (2017) documented a high prevalence of theileriosis in cattle, with 55.2% (1,074 out of 1,946 cattle blood samples) testing positive in Sepang, Selangor. Similarly, Ola-Fadunsin *et al.* (2020) reported a prevalence of 49.76% for *T. orientalis* in 1,045 cattle blood samples. Conversely, Ab Manap *et al*. (2024) reported a very low prevalence of *T. orientalis*, at only 0.81% in Mafriwal cattle.

Several factors can influence the overall prevalence of protozoa, such as the farm management system and the frequency of de-ticking of animals (Ola-Fadunsin, 2017). The first author observed that cattle raised extensively in open fields have a higher prevalence of tick-borne protozoa compared to those raised intensively in controlled environments. This is because cattle raised extensively in open fields are more exposed to tick infestations when they graze in the fields, thereby increasing the risk of acquiring *Theileria*. Similarly, cattle that are infrequently or rarely de-ticked are at a higher risk, as prolonged tick infestations can facilitate the transmission of *Theileria* and other protozoa from ticks to their hosts.

BLAST analysis in this study revealed the presence of two *Theileria* species: *Theileria orientalis* and *Theileria sinensis*. Interestingly, two of the ten positive *Theileria* samples were closely related to *Theileria annulata*. However, phylogenetic tree analyses showed that both sequences clustered with *T. orientalis* rather than *T. annulata*. This suggests that the *T. annulata* sequences in the BLAST hits may have been misidentified or could represent a different species. In this study, no *Theileria luwenshuni* was detected in the tick pools, unlike in previous reports by Kho *et al*. (2017). This absence could be due to the fact that *T. luwenshuni* is more commonly found in small ruminants, such as goats and sheep, rather than in cattle (Phipps *et al*., 2016).

No *Hepatozoon* was detected in any of the 130 tick pools in this study. This finding contrasts with a study by Tila *et al*. (2023), who reported the detection of *Hepatozoon ayorgbor* in *R. haemaphysaloides* and *Hepatozoon colubri* in *Haemaphysalis sulcata* and *Hyalomma anatolicum*, with all these tick species collected from farm ruminants in Pakistan. However, the authors noted that both *H. ayorgbor* and *H. colubri* are commonly associated with reptiles, leading to the presumption that the protozoa detected in domestic animals and ticks might have originated from wildlife. Although the risk of hepatozoonosis among cattle appears to be very low or non-existent in this study, it does not rule out the possibility that these animals could contract *Hepatozoon* protozoa from nearby wildlife such as snakes, rodents, and wild boars, or from known animal vectors such as stray dogs. Thus, the potential for cross-species transmission from wildlife to domestic animals remains a consideration, especially in areas where livestock and wildlife habitats overlap.

The absence of *Babesia* and *Theileria* in tick species other than *R. microplus* observed in this study may be due to the small sample size, rather than the actual absence of these protozoa in those ticks. *Rhipicephalus haemaphysaloides*, for instance, is known to transmit several *Babesia* and *Theileria* protozoa. Previously, Gill *et al*. (1980) demonstrated that *R. haemaphysaloides* adults could transmit *Theileria ovis* to susceptible sheep. The infected sheep subsequently developed parasitaemia and other clinical symptoms indicative of *T. ovis* infection. Similarly, Ma *et al*. (1989) reported that *R. haemaphysaloides* adults could transmit *B. bovis* to a healthy buffalo. In their study, ticks were experimentally infested on the ear of a splenectomised buffalo, and ticks were collected daily. The salivary glands of these ticks were subjected to Giemsa's stain to examine the presence of the pyriform bodies of *B. bovis*. The pyriform body was observed in the tick salivary glands on day 2 post-infection, and *B. bovis* was detected in the buffalo's peripheral blood by day 4. The infection rate of erythrocytes peaked at 9.6% on day 19 post-infection, followed by a decline. The buffalo exhibited symptoms such as high body temperature (up to 41.6°C), depression, anaemia, and icterus. Furthermore, Li *et al*. (2016) provided evidence that *B. microti* could be artificially transmitted by *R. haemaphysaloides*. The authors suggested that this tick species might be a potential vector of human babesiosis in southern China, posing a public health concern. Other *Babesia* species, such as *B. orientalis*, have also been detected in *R. haemaphysaloides* (Liu *et al*., 2007), although the vector competence of this tick for these protozoa has not been fully confirmed. Additionally, Shastri *et al*. (1988) and Hamid *et al*. (2022) have reported the detection of *Babesia naoakii* and *T. orientalis* in *H. bispinosa*, respectively, although the vector competence of this tick species remains unconfirmed. To date, no *Babesia* or *Theileria* has been reported from *Haemaphysalis wellingtoni* tick (Kazim *et al*., 2022).

The lack of detection of *Babesia* and *Theileria* in tick species other than *R. microplus* in this study does not necessarily indicate the absence of these protozoa. Further research with larger sample sizes and broader geographic coverage is needed to assess the true prevalence and distribution of these protozoa in various tick species across Malaysia.

CONCLUSION

A total of 15 pools of *R. microplus* ticks tested positive for *Babesia bigemina*, while another 10 pools were positive for *Theileria* species closely related to *T. orientalis* and *T. sinensis*. No *Hepatozoon* protozoa were found in any of the four tick species collected. Further studies are needed to explore the tripartite interactions between *Babesia* and *Theileria* in ticks and their cattle hosts.

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