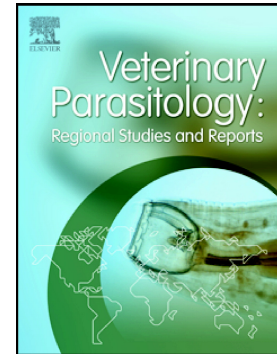


## Accepted Manuscript

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**The first molecular survey of theileriosis in Malaysian cattle, sheep and goats**

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**Abstract**

This study reports the molecular detection of *Theileria* spp. from six cattle farms, a sheep farm and a goat farm located at different states in Peninsular Malaysia. Animal blood samples were screened for the presence of *Theileria* DNA using a conventional polymerase chain reaction (PCR) assay. A total of 155 (69.2%) of 224 cattle investigated were PCR-positive for *Theileria* DNA. The occurrences of *Theileria* spp. ranged from 17.5% to 100.0% across six cattle farms. *Theileria* DNA was detected from 90.0% of 40 sheep but none of 40 goats examined in this study. Sequence analyses of amplified 18S rRNA partial fragments (335-338 bp) confirmed the identification of *Theileria buffeli*, *Theileria sergenti*, and *Theileria sinensis* in representative samples of cattle and ticks. *T. luwenshuni* was identified in the infected sheep. The high occurrences of *Theileria* spp. in our farm animals highlight the needs for appropriate control and preventive measures for theileriosis.

Keywords: *Theileria* spp., cattle, sheep, Peninsular Malaysia

## 1. Introduction

*Theileria* spp. (phylum *Apicomplexa*, order *Piroplasmida*, family *Theileriidae*, subclass *Piroplasmia*) is a tick-borne protozoan which infects a wide range of hosts, including domestic and wild ruminants (Mans et al., 2015). Severe bovine theileriosis may be caused by *Theileria annulata* and *Theileria parva*, two most pathogenic and economic important parasites in cattle (Mans et al., 2015). *Theileria orientalis* complex (*Theileria sergenti*/*Theileria buffeli*/*Theileria orientalis*), the causative agent of Oriental theileriosis, demonstrates a low pathogenicity in cattle (Chaisi et al., 2014; Mans et al., 2015; Perera et al., 2014). However, the ikeda and chitose genotypes of the complex have been reported to implicate in outbreaks of oriental theileriosis in beef and dairy cattle in the Asia-Pacific region (Aparna et al., 2011; Bawm et al., 2014; Islam et al., 2011; Izzo et al., 2010; Kamau et al., 2011; McFadden et al., 2011). The symptoms of the infected cattle include high fever, anaemia, jaundice, lethargy, and weakness (Aparna et al., 2011; Islam et al., 2011; Izzo et al., 2010). Additionally, significant milk production losses have been reported in cattle infected with *T. orientalis* (Perera et al., 2014). *Theileria sinensis*, another phylogenetically closely related members of *T. orientalis* complex, has been documented as the causative agent of bovine theileriosis in China (Bai et al., 2002). Ovine theileriosis is an important tick-borne disease of sheep caused by *Theileria lestoquardi*, *Theileria luwenshuni*, and *Theileria uilenbergi* in the tropical and subtropical regions (Yin et al., 2002).

Identification of *Theileria* parasites is essential for diagnostic, drug and vaccine development. Although blood smear microscopic examination has been used for routine diagnosis of *Theileria* infection, the method is ineffective for differentiation of *Theileria* spp. and detection of carrier animals with low parasitemia. The introduction of polymerase chain reaction (PCR)-based detection method has enabled detection of *Theileria* spp. with high

sensitivity and specificity (Mans et al., 2015). Sequence analysis of the 18S small subunit ribosomal RNA (18S rRNA) gene has been adopted as one of the promising markers for speciation of *Theileria* spp. (Chaisi et al., 2014; Mans et al., 2015).

As theileriosis may impact ruminant industry, an important component of Malaysia agriculture sector (Ariff et al., 2015), improved detection and surveillance of *Theileria* parasites is essential. Currently, there is no nationwide study on the actual occurrence and species status of the *Theileria* spp. circulating in our farm animals. In a recent Malaysian study, theileriosis has been reported as one of the important parasitic diseases in approximately 20.0% of cattle in one of the states (Zainalabidin et al., 2015), based on microscopic examination of blood smears. Using a PCR-based approach, this study was aimed to determine the occurrence and genotypes of *Theileria* spp. in Malaysian cattle, goats and sheep, and to assess the possible carriage of *Theileria* spp. in ticks.

## **2. Materials and methods**

### **2.1 Study sites and sample collection**

Permission for blood sampling was obtained from the Department of Veterinary Services (DVS), Ministry of Agriculture and Agro-based Industry, Malaysia (Reference number: JPV/PSTT/100-8/1). The study was conducted in six DVS farms located in different states (Negeri Sembilan, Pahang, Kelantan, Terengganu, Johore and Kedah, Table 1) from February to September 2013. Blood sampling was carried out by farm workers according to standard veterinary care and practice. Briefly, one to three milliliters of blood were collected from each animal via jugular vein into EDTA-coated tubes and transported on ice to the laboratory.

Whenever possible, ticks were collected using tweezers from the ear, eyes, flank, abdomen, tail and perineal regions of the animals, and preserved in -80°C freezer prior to

processing. Ticks were identified to the genus level according to Walker et al. (2003) and Geevarghese and Mishra (2011), as previously described by Kho et al. (2015).

## 2.2 GIEMSA stain

Blood smear was prepared by spreading one drop of whole blood sample on a clean labelled slide and air dried. The smear was fixed with methanol (99%) for 4 min and stained in 5% Giemsa solution (Merck, Germany) for 20 min. The stained blood smear was examined at 1000x magnification for the presence of *Theileria* spp. (Hoghooghi-Rad et al., 2011).

## 2.3 Molecular detection of *Theileria* spp.

DNA was extracted from 200 µL of animal blood samples using QIAamp DNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Ticks were first thawed, washed in 5% sodium hypochlorite and 70% ethanol and rinsed in sterile distilled water prior to homogenization using surgical blades (Duh et al., 2010). Each tick homogenate was then subjected to DNA extraction using QIAamp DNA mini kit (Qiagen, Hilden, Germany).

To rule out PCR false-negative results, a conserved region (358 bp) of the cytochrome B (*cytB*) gene of the mitochondrial DNA was amplified from each animal blood DNA sample using primers *cytB*for (5'-CCATCCAACATCTCAGCATGATGAAA-3') and *cytB*rev (5'-CCCCTCAGAATGATATTTGTCCTCA-3') and PCR condition as described by Oshaghi et al. (2006). For screening of *Theileria* DNA in the samples, PCR assays were performed using primers, Tbs-S: 5' CAC-AGG-GAG-GTA-GTG-ACA-AG-3' and Tbs-A: 5'CTA-AGA-ATT-TCA-CCT-CTG-ACA-G-3' which amplify the 18S rRNA genes of *Theileria* spp. (426-430 bp, (Ghaemi et al., 2012). PCR assays (25 µl) were performed in a Bio-Rad thermal cycler (My Cycler<sup>TM</sup>) by adding 2µl DNA template to 19.4 µl sterile distilled water, 2.5 µl

10X DreamTaq buffer, 0.5  $\mu$ l 10 mM dNTPs, 0.2  $\mu$ l of each primer (25  $\mu$ M), and 0.2  $\mu$ l DreamTaq<sup>TM</sup> DNA Polymerase (Fermentas, Lithuania). The PCR conditions included an initial denaturation step at 95°C for 5 min followed by 40 cycles of denaturation at 94°C for 45 sec, annealing at 55°C for 45 sec, and extension at 72°C for 45 sec. The PCR program was completed with a final extension step at 72°C for 5 min. A known *Theileria*-positive sample was used as positive control. All PCR products were separated on a 1.5% agarose gel at 100V for 45 minutes and visualized using a UV transilluminator (G-Box, Syngene, UK). PCR products were purified using PCR purification kit (GeneAll Expin<sup>TM</sup>) following the protocol of the manufacturer.

#### 2.4 Sequence analysis of *Theileria* spp.

Selected samples were sequenced to confirm the species status of the *Theileria* parasites. Amplicons were purified using GeneAll Expin<sup>TM</sup> Combo GP (GeneAll, Seoul, South Korea) as described by the manufacturer. Sequencing was performed with a Big Dye Terminator version 3.1 Cycle Sequencing kit (Applied Biosystems, USA) on an ABI PRISM 377 Genetic Analyzer (Applied Biosystems, USA), using forward and reverse primers. Sequence assembly and alignment were performed using BioEdit Sequence Alignment Editor Software (Version 7.0.5.3). The resulting sequences were compared with known *Theileria* sequences deposited in GenBank database using the Basic Local Alignment Search Tool (BLAST) program (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>; National Center for Biotechnology Information, Bethesda, MD).

Phylogenetic relationships among various *Theileria* species were determined based on the partial fragments of the 18S rRNA gene (335-338bp). A phylogenetic tree was constructed by using the neighbour-joining method of the MEGA6 software (Tamura et al.,

2013) and bootstrapping with 1,000 replicates. *Babesia bovis* (L31922) was used as an outgroup for comparison.

### 2.5 Nucleotide sequence accession numbers

The nucleotide sequences of *Theileria* spp. determined in this study were submitted to the GenBank database with the accession numbers (KX263951-KX263956) as shown in Table 2.

## 3. Results

Of 304 animal blood samples (224 cattle, 40 sheep, 40 goats) tested, 191 (62.8%) were positive upon amplification using the conventional PCR assays (Table 1). The occurrences of *Theileria* spp. ranged from 17.5% to 100.0% across the six cattle farms, with an average detection rate of 69.2% in cattle. *Theileria* parasites were detected from 145 (78.8%) of 184 beef cattle, and 10 (25.0%) of 40 dairy cattle. A total of 36 out of 40 (90.0%) sheep were infected with *Theileria* spp., while none of the 40 goats investigated were positive for theileriosis.

A total of 28 (14.7%) amplified fragments (selected from at least three samples from each farm) were subjected to direct sequencing, however; majority of the sequences could not be analysed due to the noisy sequence data which appeared after the first 200 nucleotides. BLAST analysis demonstrated 99-100% sequence similarity with published *Theileria* sequences for nine samples with good sequence quality. Table 1 shows the analysis of the amplified fragments (335-338 bp) obtained from each farm. Two samples from Farms 1 and 5 demonstrated the highest sequence similarity (99.7%) to *T. sergenti* Ikeda Japan (AB000271), and next closest to *Theileria* sp. type B/E (99.4%, U97048/U97053). One sample from Farm 2 demonstrated 100% sequence similarity to *Theileria* sp. type C (U97051) and next closest (99.1%) to *T. buffeli* Warwick-Australia (AB000272). One sample



from Farm 4 demonstrated 100% sequence similarity to *T. buffeli* Warwick-Australia (AB000272). Two samples derived from dairy cattle in Farm 6 demonstrated the closest sequence similarity (99.4%) to *Theileria* sp. type D (U97052) and next closest to *T. sinensis* from cattle in China (EU277003). Three representative samples derived from sheep (Farm 7) demonstrated 100.0% sequence similarity to *T. luwenshuni* which had been identified in sheep from China (KC414096). Figure 1 shows the intraerythrocytic infections of *Theileria* spp. in the smears of PCR-positive blood samples.

*Haemaphysalis bispinosa* (n=27) and *Rhipicephalus microplus* (n=41) ticks collected from three *Theileria*-positive cattle farms (Farms 1, 2 and 3) were subjected to PCR screening for *Theileria* spp. Seven (10.3%) of the cattle ticks (three *R. microplus* and four *H. bispinosa*) from two cattle farms (Farms 2 and 3) were positive for *Theileria* DNA. Sequence analysis of the partial fragment of the 18S rRNA gene of *Theileria* spp. in a *R. microplus* tick from Farm 2 reveals 99.7% similarity to *Theileria* sp. type C (U97051), while the sequence derived from a *H. bispinosa* tick in Farm 3 demonstrates 99.7% sequence similarity to that of *T. buffeli* Warwick-Australia (AB000272).

Figure 2 is a dendrogram showing the phylogenetic relationship of the Malaysian *Theileria* spp. with other *Theileria* reference strains. Low bootstrap value (43.0%) was noted between *T. orientalis* complex (*T. buffeli*/*T. sergentii* and type A, B, B1, C, E, H), suggesting the closely relatedness of the members in *T. orientalis* complex. *T. sinensis* and *T. luwenshuni* were segregated at different branches of the tree, exhibiting higher bootstrap values (62.0% and 98.0%, respectively) in the dendrogram.

#### 4. Discussion

Based on PCR findings in this study (Table 1), it appears that theileriosis is widespread in our cattle, with as high as 100% *Theileria* detection rate being reported in two

farms. The molecular evidence of theileriosis caused by *T. buffeli* (Warwick), *T. sergenti* (Ikeda type) and *T. sinensis* in Malaysian cattle was presented for the first time in this study. *T. buffeli* (Warwick) has a wide distribution in cattle and buffaloes in many parts of the world (Kamau et al., 2011). *T. sergenti* (Ikeda type) is commonly found in anemic cattle suffering from bovine piroplasmiasis in Japan and Korea (Kamau et al., 2011). The isolation of a *T. sergenti* closely related strain (*Theileria* type Ipoh) has been previously reported in Malaysia (Chansiri et al., 1998). *T. sinensis* which was only detected in a dairy cattle in this study, has been reported in cattle from the central part of Gansu Province, China (Bai et al., 2002). The similar genotype distribution of *Theileria* parasites in Malaysia and other studies (Bawm et al., 2014; Chaisi et al., 2014; Bai et al., 2002; Perera et al., 2014) may be caused by the movement of animals carrying *Theileria* parasites in the Asia-Pacific region.

A high prevalence of theileriosis was noted in beef cattle (78.8%), as compared to dairy cattle (25.0%) investigated in this study. The reason for the difference observed between the two groups of cattle is not clear as only a small sample size of dairy cattle was tested, as compared to meat cattle. Environmental factors such as geographic variation, the immunity of the cattle and their exposure to different types and distribution of tick vectors may influence the prevalence of theileriosis. Further investigation to verify this finding is thus warranted.

This study also reports a high rate (90.0%) of ovine theileriosis in a flock of sheep outside China where the species was first described. The predominant *Theileria* spp. identified in this study, *T. luwenshuni*, has also been reported with disease and mortality in sheep from the United Kingdom (Phipps et al., 2016). It is yet to be investigated whether *T. luwenshuni* is indigenous to Malaysia or could have been introduced from other geographical regions.

The circulation of tick vectors plays an important role in the spread of theileriosis. Various species of *Haemaphysalis* ticks have been implicated as the vectors for *Theileria* spp. For instance, *T. sergenti* is transmitted by *Haemaphysalis longicornis* (Yin et al., 2002), while *T. sinensis* is transmitted by *Haemaphysalis qinghaiensis* (Bai et al., 2002). The detection of *Theileria* spp. in our cattle ticks (*R. microplus* and *H. bispinosa*) implies the potential of the ticks in the transmission of theileriosis.

Using PCR-based method, we detected a higher rate of *Theileria* parasites in cattle, as compared to a previous Malaysian study (Zainalabidin et al., 2015). Apart from difference in cattle population, it is believed that the insensitivity of the microscopic examination method may contribute to the lower detection rate (20%) as determined in the previous study. The conventional classification of members in the *T. orientalis* complex which is largely based on phenotypic features is challenging due to the similarities in the morphology, serology, life cycle and vector transmission (Uilenberg et al., 1985). Identification of *Theileria* parasites can become even more complicated with the occurrence of mixed or co-infections of *Theileria* spp. in the farm animals. Mixed infection of *Theileria* spp. is suspected in this study as the sequence data generated for a majority of *Theileria*-amplified fragments was unsatisfactory for further analysis. In fact, mixed populations of different *Theileria* types have been reported in Japan (Kubota et al., 1995), Korea (Baek et al., 2002) and Thailand (Sarataphan et al., 2003). For verification of the current finding, cloning of amplified fragments or use of allele-specific PCR assay should be attempted for differentiation of *Theileria* spp. in these samples.

## 5. Conclusion

The findings in this study suggest a potential threat of bovine and ovine theileriosis to the ruminant industry and highlight the needs for appropriate control and preventive

measures. As genetic variation among *Theileria* parasites could be linked with host specificity and virulence in the parasites, more extensive investigation is required to provide information on the clinical aspects of the infected animals.

## ACKNOWLEDGEMENT

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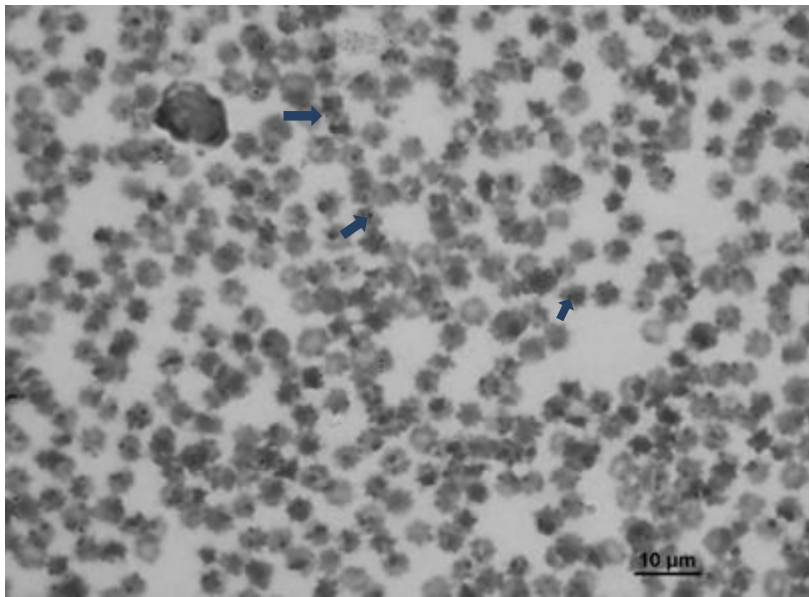
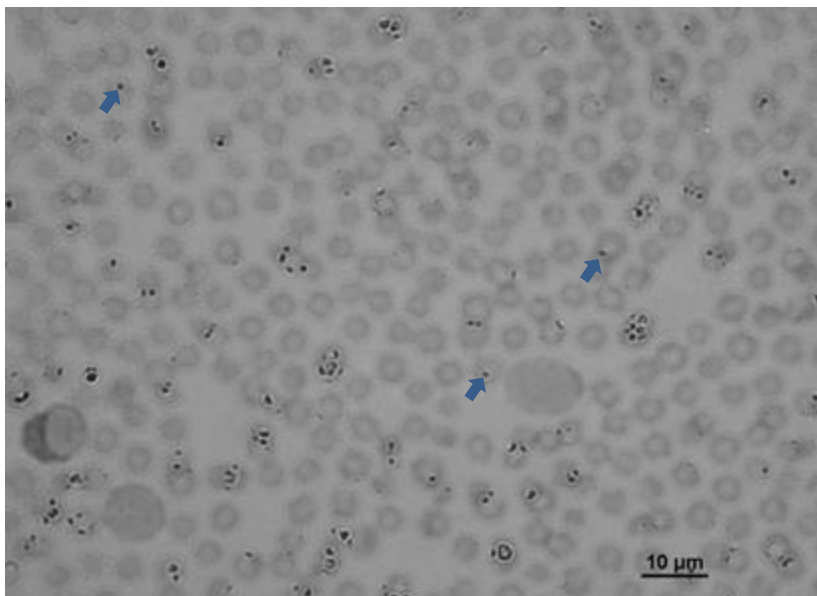
**Table 1.** Detection of *Theileria* spp. in cattle, sheep and goat samples collected from eight farms in Peninsular Malaysia.

**Table 2.** *Theileria* spp. identified from this study.

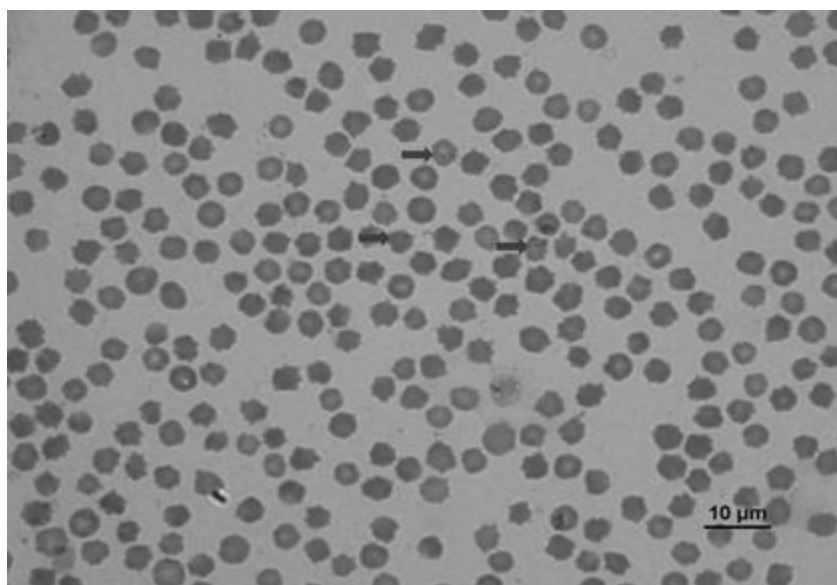
**Fig 1.** Microscopic examination of blood samples obtained from:

- i. Cattle infected with *Theileria* sp. Y4955
- ii. Cattle infected with *Theileria* sp. I72-4598
- iii. Sheep infected with *Theileria* sp. KN-95

**Fig 2.** Phylogenetic tree constructed by the neighbor-joining method based on 18S rRNA gene sequences showing the relationship of *Theileria* species (Table 2). *Babesia bovis* (GenBank accession no.: L31922) was used as an outgroup. The bootstrap percentage values are given at the nodes of the tree. Scale bar = 0.02 units.

**(i)****(ii)****(iii)**

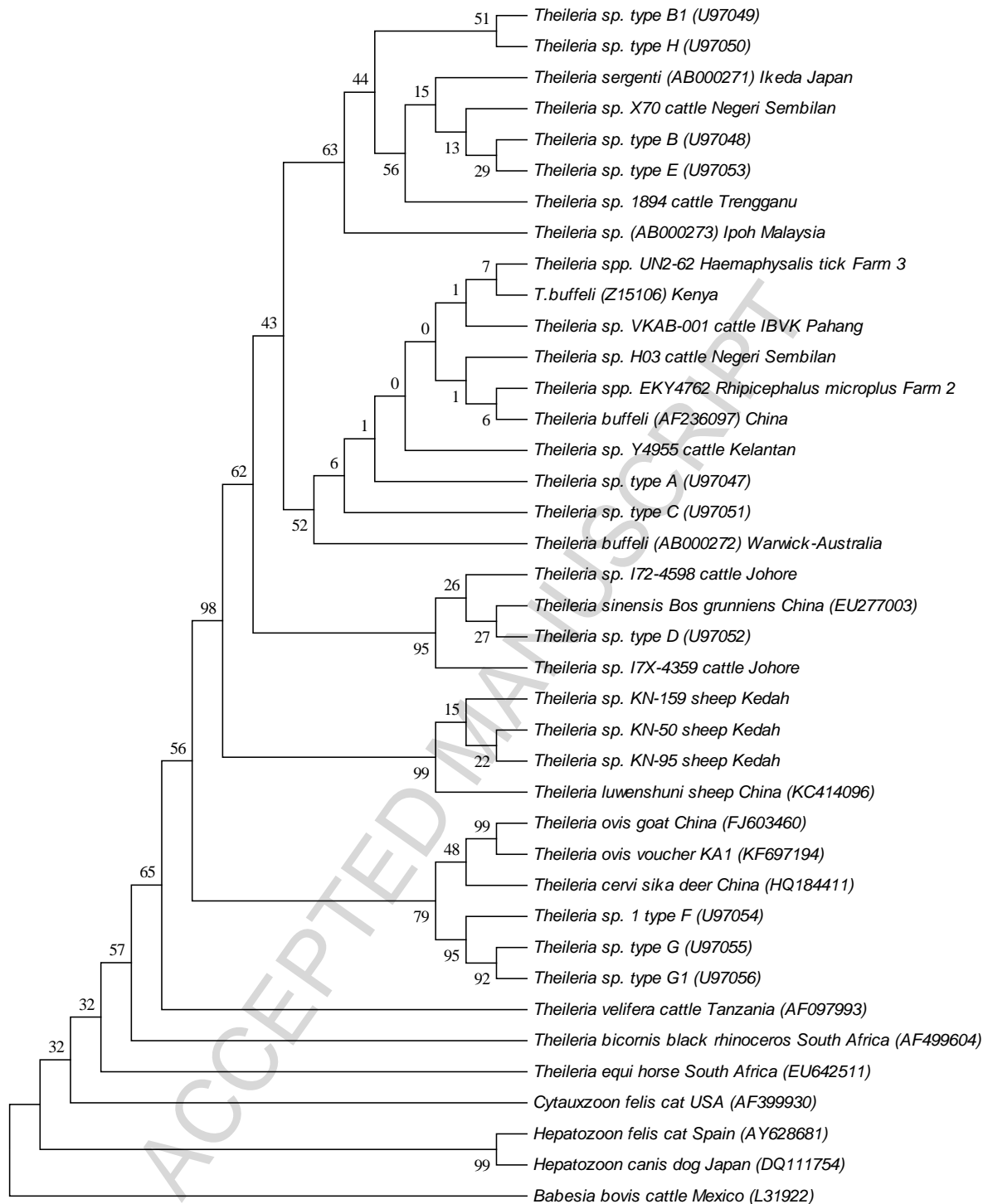
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**Figure 1.** Microscopic examination of blood samples obtained from:

- i. Cattle infected with *Theileria* sp. Y4955
- ii. Cattle infected with *Theileria* sp. I72-4598
- iii. A sheep infected with *Theileria* sp. KN-95





**Fig 2.** Phylogenetic tree constructed by the neighbour-joining method based on 18S rRNA gene sequences showing the relationship of *Theileria* species (Table 1). *Babesia bovis* (GenBank accession no.: L31922) was used as an outgroup. The bootstrap percentage values are given at the nodes of the tree. Scale bar, 0.02 units.

**Table 1.** Detection of *Theileria* spp. in 304 cattle, sheep and goat blood samples collected from eight farms in Peninsular Malaysia

States	Farms (Animal breed, n)	No. (%) PCR-positive blood samples
Negeri Sembilan	Farm 1 - Beef cattle (Nellore, 11; YKK, 15)	26 (100.0)
Pahang	Farm 2 - Beef cattle (Kedah-Kelantan, 38; )	37 (97.4)
	Farm 3 - Beef cattle (Nellore, 32; Brahman , 8)	35 (87.5)
Kelantan	Farm 4 - Beef cattle (Kedah-Kelantan, 40)	40 (100.0)
Terengganu	Farm 5 - Beef cattle (YKK, 40)	7 (17.5)
Johore	Farm 6 - Dairy cattle (Jersey, 4; Mafriwal, 36)	10 (25.0)
Kedah	Farm 7 - Sheep (Damara, 40)	36 (90.0)
Negeri Sembilan	Farm 8 - Goat (Boer, 32; Savannah, 2; African dwarf, 5; Cashmere, 1)	0 (0.0)
Total		191 (62.8%)

All the cattle and sheep farms were managed by rotational grazing system while goats were kept under zero grazing practice.

YKK- Yellow cattle cross Kedah-Kelantan

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**Table 2.** *Theileria* spp. identified from this study.

Species	Host (breed)	Age	Origin	GenBank accession no.	Reference strains with most matching sequence (% sequence similarity, GenBank accession no.)
<i>Theileria</i> sp. X70	Cattle (Nellore)	27 months	Farm 1, Negeri Sembilan	KX263951	<i>T. sergentii</i> Ikeda Japan (99.7%, AB000271), <i>Theileria</i> sp. type B/E (99.4%, U97048/U97053)
<i>Theileria</i> sp. VKAB-001	Cattle (Kedah-Kelantan)	6 months	Farm 2, Pahang	Same as U97051	<i>Theileria</i> sp. type C (100%, U97051), <i>T. buffeli</i> Warwick Australia (99.1%, AB000272 )
<i>Theileria</i> sp. Y4955	Cattle (Kedah-Kelantan)	22 months	Farm 4, Kelantan	KX263952	<i>T. buffeli</i> Warwick Australia (100%, AB000272)
<i>Theileria</i> sp. 1894	Cattle (YKK)	16 months	Farm 5, Terengganu	Same as KX263951	<i>T. sergentii</i> Ikeda Japan (99.7%, AB000271), <i>Theileria</i> sp. type B/E (99.4%, U97048/U97053)
<i>Theileria</i> sp. I7X-4359	Cattle (Mafriwal)	36 months	Farm 6, Johore	KX263953	<i>Theileria</i> sp. type D (99.4, U97052), <i>T. sinensis</i> China (99.1%, EU277003)
<i>Theileria</i> sp. I72-4598	Cattle (Mafriwal)	22 months	Farm 6, Johore	Same as KX263953	<i>Theileria</i> sp. type D (99.4, U97052), <i>T. sinensis</i> China (99.1%, EU277003)
<i>Theileria</i> sp. KN-95	Sheep (Damara)	16 months	Farm 7, Kedah	KX263954	<i>T. luwenshuni</i> (100%, KC414096)
<i>Theileria</i> sp. KN-159	Sheep (Damara)	15 months	Farm 7, Kedah	Same as KX263954	<i>T. luwenshuni</i> (100%, KC414096 )
<i>Theileria</i> sp. KN-50	Sheep (Damara)	16 months	Farm 7, Kedah	Same as KX263954	<i>T. luwenshuni</i> (100%, KC414096)
<i>Theileria</i> spp. EKY4762	<i>Rhipicephalus microplus</i> tick	-	Farm 2, Pahang	KX263956	<i>Theileria</i> sp. type C (99.7%, U97051)
<i>Theileria</i> spp. UN2-62	<i>Haemaphysalis bispinosa</i> tick	-	Farm 3, Pahang	KX263955	<i>T. buffeli</i> Warwick Australia (99.7%, AB000272)

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### Highlights

- Animal blood samples were screened for *Theileria* DNA using PCR assays.
- The occurrences of *Theileria* ranged from 17.5% to 100.0% across six cattle farms.
- *Theileria* DNA was detected from 90.0% of 40 sheep but none from the goats.
- *T. buffeli*, *T. sergenti*, and *T. sinensis* were identified in cattle by sequencing.
- *T. luwenshuni* was identified in the infected sheep.

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