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Research Article

Journal of Veterinary and Animal Sciences
ISSN (Print): 0971-0701, (Online): 2582-0605
https://doi.org/10.51966/jvas.2024.55.4.762-770

Molecular detection of *Theileria orientalis* genotypes in cattle ticks from Kerala, India[#]

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Citation: Prathyusha, S., Radhika, R., Lakshmanan. B, Syamala, K., Marykutty, T., Justin, K. D., Athira C. P. and Devada, K. 2024. Molecular detection of *Theileria orientalis* genotypes in cattle ticks from Kerala, India. *J. Vet. Anim. Sci.* **55** (4):762-770

Received: 05.09.2024 Accepted: 28.10.2024 Published: 31.12.2024

Abstract

Ticks are obligate haematophagous ectoparasites affecting cattle and act as potential vector for haemoprotozoan diseases like oriental theileriosis caused by Theileria orientalis, leading to substantial economic losses to the dairy farmers. This study aimed to assess the prevalence of tick species and to detect T. orientalis and its genotypes in ticks collected from cattle across north, central and south zones of Kerala, India. A total of 656 ticks were collected and identified as Haemaphysalis bispinosa (40.1 per cent), Rhipicephalus annulatus (32.16 per cent), R. microplus (18.44 per cent) and H. intermedia (9.3 per cent). Haemaphysalis bispinosa was the primary tick species infesting cattle in Kerala. Primers specific to the T. orientalis major piroplasm surface protein (MPSP) gene amplified a 776 bp PCR product in four tick pools of R. annulatus. Genotype-specific PCR targeting MPSP gene confirmed the Buffeli genotype in one pool and mixed genotypes (Chitose and Buffeli) in two pools. One positive tick pool failed to amplify with Chitose, Buffeli or Ikeda genotype specific primers. Furthermore, sequencing and phylogenetic analysis of this pool with T. orientalis MPSP gene sequences revealed close similarity with type 7, belonging to pathogenic Ikeda strain. This study has underscored the importance of circulation of pathogenic genotypes in Kerala. These findings thus highlighted the need for effective tick control strategies to mitigate the spread of T. orientalis, particularly in tropical regions like Kerala.

Key words: *Tick-borne pathogens, nucleotide sequence, phylogeny, T. orientalis, R. annulatus*

Oriental theileriosis, a tick-borne parasitic disease affecting cattle and buffaloes, caused by *T. orientalis* poses a significant global threat to livestock. In India, *Theileria annulata* causes tropical theileriosis, leading to economic losses of around US$1295 million annually (Narladkar, 2018). Unlike other *Theileria* species like *T. annulata* and *T. parva*, which target lymphocytes, *T. orientalis* primarily multiplies in red blood cells, resulting in anaemia and related complications (George *et al*., 2015). This proliferation leads to the rupture of red blood cells, subsequently causing anaemia (Watts *et al*., 2016). *Theileria orientalis* infections in cattle and buffaloes have been documented in different states across India in recent years with reported mortalities, especially from Kerala, Andhra Pradesh, Assam, Odisha, Telangana, Himachal Pradesh and Uttar Pradesh (Aparna *et al*., 2011; Vinodkumar *et al*., 2016; George *et al*., 2015; Kakati *et al*.,

[#]*Part of PhD thesis submitted to Kerala Veterinary and Animal Sciences University, Pookode, Wayanad, Kerala*

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2015; Baghel *et al*., 2023; Patial *et al*., 2021). Among the Ixodid ticks, *Haemaphysalis* spp. and *Rhipicephalus* spp. act as potential vectors of *T. orientalis* in India (Altangerel *et al*., 2011). Even though oriental theileriosis has emerged as a virulent pathogen in different states of India with reported mortality in cattle and buffaloes, there have been no previous reports to detect *T. orientalis* genotypes in ticks. This study aims to address that gap by examining the distribution of tick species across Kerala and molecularly identifying *T. orientalis* and its genotypes in ticks from cattle. The findings offer valuable insights into the epidemiology of the disease and emphasise the need for effective tick control strategies to mitigate the spread of theileriosis in Kerala's cattle population. Consequently, this study presents the detection of *T. orientalis* genotypes in ticks using genotype specific PCR for the first time in the state.

Materials and methods

Study area and sample collection

In the present study, a total of 578 cattle were examined for tick infestation in six districts belonging north, central and south zones of Kerala. The ticks were collected manually from the infested animals and transferred to glass vials containing 70 % ethanol. The collected ticks from different zones of Kerala are given in Table 1.

Morphological identification

Ticks were examined using a stereo zoom microscope (Leica Microsystems GmbH, Germany) and identified to the species level using the taxonomic keys of Sen and Fletcher (1962) and Walker *et al*. (2003). After identification, the ticks were pooled according to their species from the same host animal into separate vials for molecular examination.

Extraction of DNA from ticks

DNA was extracted from the ticks collected from cattle using the salting out method (Jain *et al*., 2017). A final volume of 100 µL DNA was eluted. The concentration, purity and quality of extracted blood DNA samples were analysed using a nanodrop spectrophotometer (Nano 2000 UV- Vis, USA). The purity of DNA was measured at a ratio of 260/280 nm wavelength and the extracted DNA was stored at -20°C until further use.

PCR for T. orientalis and its genotypes

The gradient cycling conditions for

Table 1. Collection of ticks from different zones of Kerala

Zone	District	Place	No. of ticks collected	Total ticks/ zone
Central	Thrissur	Chirakkakode	35	387
		Kodaly	32	
		Kodakara	25	
		Vellikulangara	27	
		Kodungalloor	28	
		Minalur, Wadakkanchery	15	
		Perinchery	19	
		Chavakad	56	
		Cherpu	29	
		Kurinjakkal	16	
		Kuttur	8	
		Chevoor	39	
	Ernakulam	Perumbavoor	26	
	Palakkad	Potundy	32	
North	Wayanad	University farm Pookode	49	155
		Meppadi	26	
		Mundakkai	37	
		Kalpetta	15	
	Kozhikode		28	
South	Alleppey	Cherthala	34	114
		Varanad	37	
		Mayithara	14	
		Thuravoor	29	
Grand total				**656**

T. orientalis were initially standardised using a known positive blood DNA sample, which exhibited heavy parasitaemia during microscopic examination of stained blood smears. Protocols for *T. orientalis* genotypes (Chitose, Ikeda and Buffeli) were separately optimised using a gradient thermal cycler (Bio-RAD T100™, USA). The species-specific primers, forward primer F 5'- CTTTGCCTAGGATACTTCCT- 3' and reverse primer R 5'- ACGGCAAGTGGTGAGAACT- 3' (Ota *et al*., 2009), which amplifies a 776 bp fragment of *MPSP* gene of *T. orientalis* were utilized. The primers used for amplification of *T. orientalis* genotypes targeting the *MPSP* gene in genotype-specific PCR are given in Table 2. The reaction was carried out in a total volume of 12.5 µL containing 2 µL genomic DNA, 6.25 µL of master mix (Green dye PCR master mix (2x), TaKaRa Emerald, Japan), 2.25 µL of nucleus-free water and 1 µL (10 pmol) of each forward and reverse primers. The PCR was optimised at an initial denaturation of 94°C for 3 min, followed by 34 cycles of 94 °C for 30 s, annealing at 59 °C for 45 s and 72 °C for 30 s with a final extension of 72 °C for 5 min for *T. orientalis*. Entire protocol was the same except an annealing temperature of 57 °C, standarised for amplification of *T. orientalis* genotypes.

A total of 3 µL of PCR product was subjected to gel electrophoresis on 2 % gel containing 0.5 µg/mL of ethidium bromide in a 1X TAE buffer. Electrophoresis was conducted at 70 volts for 60 min. After electrophoresis, the gel was transferred to a UV transilluminator (GeNeiTM, Bengaluru) for band visualisation and documented using a Gel Documentation System (Bio-rad, USA).

Nucleotide sequence analysis

The amplicons obtained from PCR were purified and sequenced at Gene Spec Biosciences, Kochi using Sanger's dideoxy nucleotide chain termination method. Bidirectional sequencing was performed with both forward and reverse primers. The sequences were aligned using EMBOSS (www.bioinformatics.nl>cgi-bin>merger) and analysed for similarity with other published sequences in online databases using the NCBI BLAST tool (www.blast.ncbi.nlm.nih.gov>blast).

Construction of phylogenetic tree

A phylogenetic tree was constructed from *T. orientalis MPSP* gene sequence from the tick pool in this study along with other sequences reported in the GenBank database, to determine the homology of the *T. orientalis* with those previously reported. This analysis was conducted using MEGA XI software Version 11.0.13, with a bootstrap value of 1000. The nucleotide sequences were aligned with Muscle and the evolutionary history was inferred using Maximum Likelihood method and the Tamura 3 parameter model. A discrete gamma distribution was applied to model evolutionary rate differences among sites.

Results and discussion

Prevalence of ticks

The different species of ticks were categorised based on their morphological features. Ticks of the genus *Rhipicephalus* were brevirostrate with an inornate scutum and possess a sub-hexagonal basis capitulum. Male ticks possessed well-developed ventral plates. *Rhipicephalus microplus* had a short rostrum, the first coxa had a hump on the dorsal border (Fig. 1a) and was notched ventrally with absence of festoons and presence of caudal process in males (Fig. 1b). But in case of *R. annulatus*, caudal process was absent (Fig. 2). Moreover, in *R. annulatus*, posterior border of adanal plates was squared with indistinct spurs, while in *R. microplus*, posterior border of adanal plates was rounded with indistinct spurs. These could be well differentiated from *R. haemaphysaloides* in which adanal shields in males were sickle-shaped with external and posterior margins forming a regular curve (Berry, 2017). None of the species were *R.hamaphysaloides.*

Ticks of the genus *Haemaphysalis* exhibited a prominent lateral projection on second palpal segment, which extended beyond the basis capitulum with its straight lateral margins. In male ticks, the ventral plates were absent. *Haemaphysalis* spp. is mainly distinguished based on number and arrangement of infra internal setae. In case of *H. bispinosa*, infra internal setae were distinctly separate, numbering four to five (Fig. 3a and 3b) and in the case of *H. intermedia* the number varied between six

Table 2. Primers for the amplification of *T. orientalis* genotypes

Genotypes of *T. orientalis*		Primer sequence	Amplicon size (bp)	Reference
MPSP gene (partial) of *T. orientalis* genotypes	Chitose (Type 1)	F 5'-GCGGATCCTCATCGTCTCTGCAACT-3'	831	Kubota *et al*. (1996)
		R 5'-TGTGAGACTCAATGCGCCTA-3'		
	Ikeda (Type 2)	F 5'-AAGGATCCGTCTCTGCTACCGCCGC-3'	826	
		R 5'-TGTGAGACTCAATGCGCCTA-3'		
	Buffeli (Type 3)	F 5'-GCGGATCCGCTCTGCAACCGCAGAG-3'	825	
		R 5'-TGTGAGACTCAATGCGCCTA-3'		

Table 3. Morphological identification of ticks collected from cattle

Zone	District	Place	No. of Ticks collected	Species of tick s identified
Central	Thrissur	Chirakkakode	35	*R. annulatus*
		Kodaly	32	*R. annulatus*
		Kodakara	15	*H. bispinosa*
		Vellikulangara	27	*R. annulatus*
		Kodungalloor	28	*H. bispinosa*
		Minalur (Wadakkanchery)	25	*R. annulatus*
		Perinchery	19	*H. bispinosa*
		Chavakad	56	*H. intermedia* (37)
				R. microplus (19)
		Cherppu	29	*H. bispinosa*
		Kurinjakkal	16	*H. intermedia*
		Kuttur	8	*H. intermedia*
		Chevoor	39	*H. bispinosa* (26)
				R. microplus (13)
	Ernakulam	Potundy	26	*R. microplus*
	Palakkad		32	*H. bispinosa*
Total			387	
North	Wayanad	University farm Pookode	49	*R. annulatus*
		Meppadi	26	*H. bispinosa*
		Mundakkai	37	*H. bispinosa*
		Kalpetta	15	*R. annulatus*
	Kozhikode		28	*R. annulatus*
Total			155	
South	Alleppey	Cherthala	34	*R. microplus*
		Varanad	37	*H. bispinosa*
		Mayithara	14	*H. bispinosa*
		Thuravoor	29	*R. microplus*
Total			114	
Grand total			656	

Table 4. Nucleotide sequence of *T. orientalis MPSP* gene (partial) in tick**s**

T. orientalis MPSP gene sequence (partial) in ticks
TACAGTCAGCGCAACCAACGCCAACGACGTCGTTTTTACTGCCGAGGATGGATACCGCTTCAAGACTCTCAAGGTTG GAGATAAGACCCTGTATACCGTAGATACATCCAAATTCACTCCAACTGTCGCCCACAGACTGAAGCATGATGAAGACC TGTTCTTCAAGCTCAACCTGTCCCACGCCAAGCCCCTTCTGTTCAAGAAGAAGAGCGACAAGGATTGGGTACAGTTT AACTATGCCCAGTACCTAGAGGATGTCCTATGGAAAGAGAAGAAGGAAAAGAAAGACCTCGATGTGTCGAAGTTCT CAGACGCAGGTCTTTTCGCCGCTGAAGCCTTTGGTACCGGAAAG

to seven and arranged close together in a feathery pattern (Fig. 4a and 4b).

Out of 578 cattle examined, 176 were found to be infested with ixodid ticks in three zones of Kerala. A total of 656 ticks were collected. The identified tick species encountered in the order of prevalence in this study included *Haemaphysalis bispinosa* (n= 263, 40.1 %), *Rhipicephalus annulatus* (n= 211, 32.16 %), *Rhipicephalus microplus* (n= 121, 18.44 %) and *Haemaphysalis intermedia* (n= 61, 9.3 %). The species identification of ticks is given in Table 3. These findings highlighted the need for targeted tick control strategies to manage the risk of tick-borne diseases, considering the specific prevalence and distribution of the different tick species. *Haemaphysalis bispinosa* was recorded across all three sampling zones, consistent with the findings of Prakasan and Ramani (2007), who identified it as the most common tick species in cattle throughout Kerala. Similarly, *H. bispinosa* was predominant in cattle from Andhra Pradesh, with higher prevalence in wet and dry zones with increased humidity compared to semi-arid regions (Kandi *et al*., 2022). Likewise, *Haemaphysalis* spp. was also the most prevalent tick species in cattle in the Gadag district of Karnataka (Krishnamoorthy *et al*., 2023). In contrast, Shyma *et al*. (2013) found *R. annulatus* to be the most prevalent species in cattle and buffaloes in the north zone of Kerala, followed by *H. bispinosa.* Studies conducted in the north, central and south regions of Kerala had

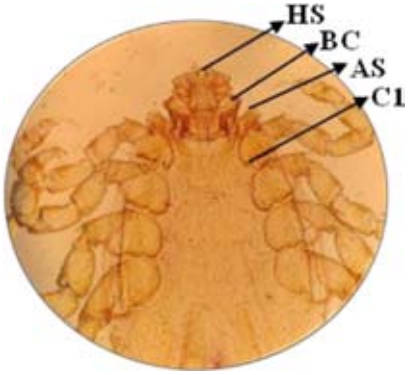


Fig 1a. *R. microplus*-ventral view

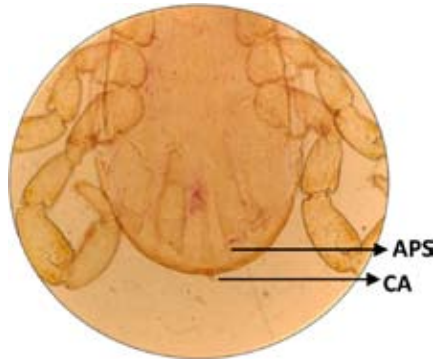


Fig 1b. *R. microplus*- posterior view

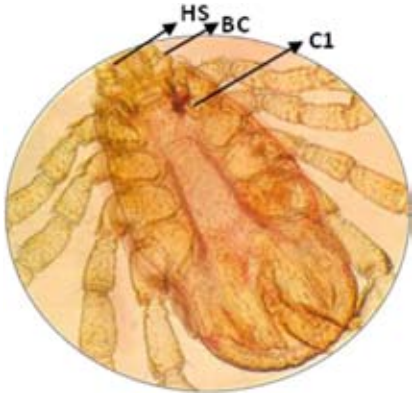


Fig 2. *R. annulatus*- ventral view

BC - Basis capitulum
C1 - Coxal spur 1
AS - Anterior spur
APS - Adanal plate spur
CA - Caudal appendage
HS - Hypostome

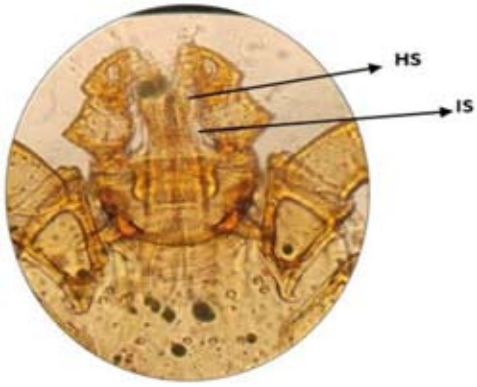


Fig. 3a *H. bispinosa*-anterior view

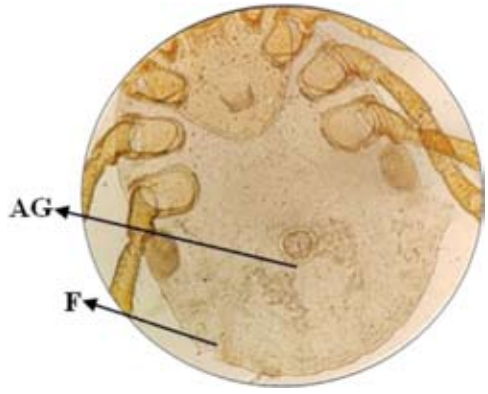


Fig. 3b *H. bispinosa*-ventral view

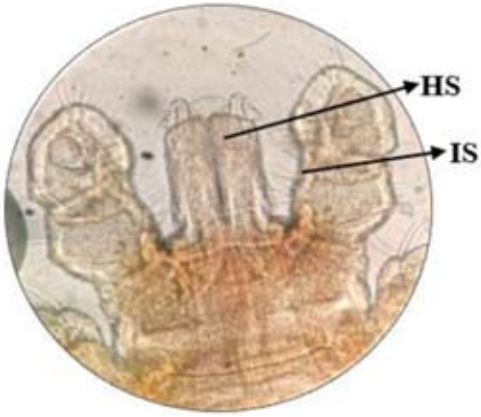


Fig. 4a *H. intermedia*-anterior view

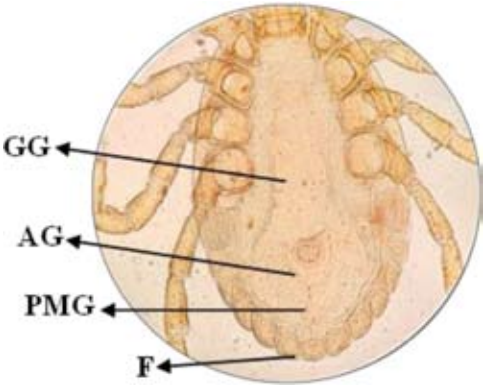


Fig. 4b *H. intermedia*-ventral view

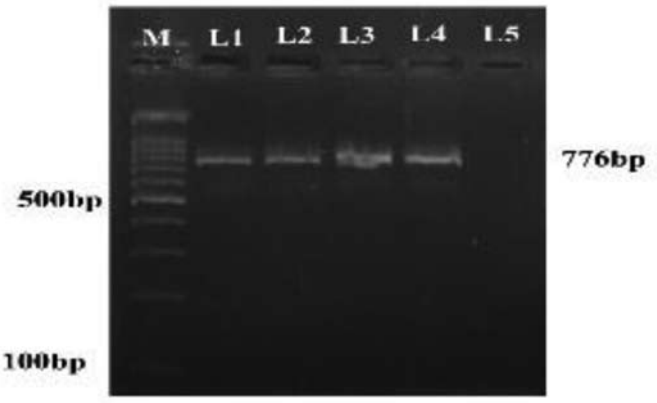
VS - Ventral spur
HS - Hypostome
C1 - Coxal spur 1
AG - Anal Groove
F - Festoon
PMG - Posterio Median Groove
IS - Infrainternal setae

confirmed that *R. annulatus* had the highest prevalence, followed by *H. bispinosa* (Nimisha *et al*., 2019; Hembram *et al*., 2022).

Molecular detection of T. orientalis in ticks

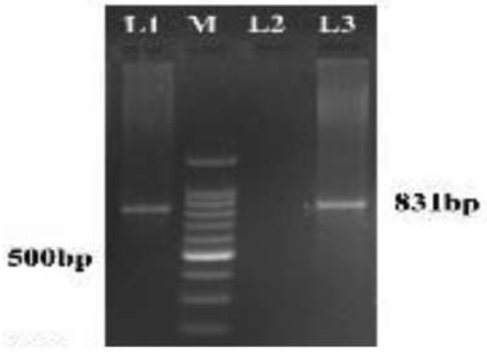
A specific 776 bp amplicon indicating positive for *T. orientalis* was observed in four tick DNA pools belonging to the *R. annulatus* species (Fig. 5). Out of four tick pools tested, one yielded a product of 825 bp, confirming the presence of *T. orientalis* Buffeli genotype. Two pools yielded mixed genotypes with products of 831 bp (Fig. 6) indicating Chitose genotype and 825 bp (Fig. 7), indicating Buffeli genotypes.

In the present study, four pools of *R. annulatus* ticks collected from cattle were confirmed to be *T. orientalis* using PCR targeting *MPSP* gene. These positive tick samples were collected from Thrissur district which is the central zone of Kerala. *Rhipicephalus annulatus* ticks



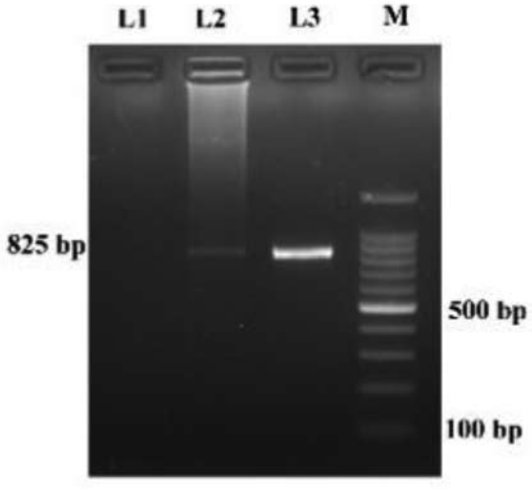
Lane M: 100 bp ladder
L2, L3: Positive amplicons
L1: NTC

Fig 5. Amplicons of *MPSP* gene of *T. orientalis* in ticks



Lane M: 100 bp ladder
L1-L4: Positive amplicons
L5: NTC

Fig 6. Amplicons of *MPSP* gene of *T. orientalis* Chitose genotype in ticks



Lane M: 100 bp ladder
L1, L3: Positive amplicons
L2: NTC

Fig 7. Amplicons of *MPSP* gene of *T. orientalis* Buffeli genotype in ticks

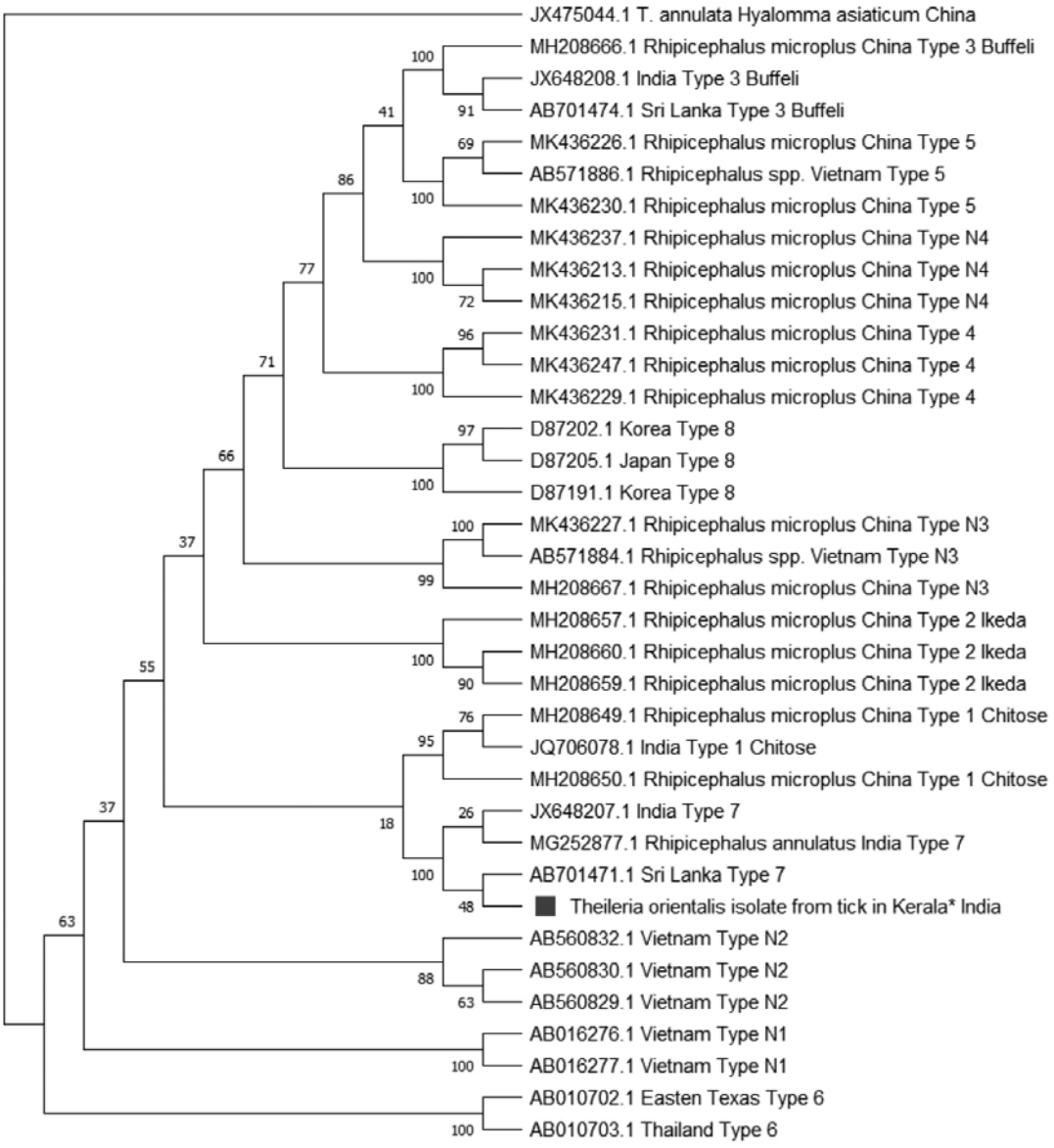


Fig 8. Phylogenetic tree illustrating the genetic relationship of the *MPSP* gene sequence of *T. orientalis* from ticks in the present study was constructed. The sample from the present study is highlighted in a blue box.

was the second most prevalent tick species on cattle, following *H. bispinosa*. However, the current study clearly identified the presence of *T. orientalis* in *R. annulatus*, a one-host tick, which may potentially serve as a vector for *T. orientalis* transmission, as these ticks could have ingested *T. orientalis* parasites along with the blood meal. *Theileria orientalis* was detected in engorged adult *R. annulatus* ticks by Nimisha *et al*. (2019) targeting the *MPSP* gene in Kerala. The present study also targeted the same *MPSP* gene.

Similarly, *T. orientalis* was amplified in six engorged *R. annulatus* ticks using the same *MPSP* primers. However, none of their egg masses or larvae exhibited amplification. Although *T. orientalis* was detected in engorged *R. annulatus* ticks, the absence of the parasite in the egg masses and larvae ruled out the possibility of trans-ovarian transmission (Hembram *et al*., 2022). In contrast, Kakati *et al*. (2015) confirmed the presence of *T. orientalis* DNA in the eggs of *R. microplus* ticks collected from infected cattle, suggesting that *R. microplus* could be a vector for the transmission of parasites through trans-ovarian mode. Similar findings were reported for *R. microplus* in Thailand (Poolkhetkit *et al*., 2015) and Vietnam (Khukhuu *et al*., 2011), as well as for *Haemaphysalis longicornis* in Australia (Hammer *et al*., 2015) and *Rhipicephalus decoloratus* and *Rhipicephalus evertsi* in Ethiopia (Kumsa *et al*., 2013).

One *T. orientalis* positive tick pool did not reveal any product corresponding to these two genotypes and was sent for sequencing for further genotype identification. This signifies the importance of other genotypes circulating in Kerala. Genotype specific PCR results showed that

ticks in Hokkaido predominantly carried *T. orientalis* type 2, whereas those in Okinawa mainly harboured type 1 from Japan, as reported by Yokoyama *et al*. (2012). *Haemaphysalis longicornis* ticks from cattle harboured *T. orientalis* DNA, specifically genotypes Ikeda, Chitose and Buffeli from Australia (Hammer *et al*., 2015).

A 776 bp sequence obtained with PCR using *T. orientalis* specific primers was sequenced (Table 4). The partial sequence of *MPSP* gene revealed 100 per cent similarity with corresponding published sequences of *T. orientalis* from different countries (MK874825.1, LC438488.1, LC438487.1, MH697845.1, MH697844.1, MH447962.1, PP409590.1, OR513426.1, OR513425.1, OR513424.1, PP395621.1, PP395620.1, PP395619.1, PP297116.1, PP297115.1). The sequence obtained was submitted to GenBank through bankit and assigned an accession number, PQ412992.

Phylogenetic analysis

The analysis involved 36 nucleotide sequences comprising isolates from the current study alongside previously published sequences from GenBank. The corresponding gene sequence of *T. annulata* from *Hyalomma asiaticum* was used as an outgroup for analysis (Fig. 8). Phylogenetic analysis in the present study revealed that the *MPSP* gene sequence of *T. orientalis* from ticks was closely related to type 7 of *T. orientalis*. This finding corroborated with the results of Nimisha *et al*. (2019), who reported that *T. orientalis* found in *R. annulatus* infesting cattle was closely related to type 7 isolate, which belonged to the pathogenic Ikeda group. This further confirms the prevalence of pathogenic Ikeda strain among cattle in Kerala. In another study by Li *et al*. (2020), phylogenetic analysis of the *18S rRNA* gene and *MPSP* gene had identified seven known genotypes (types 1–5, 7 and N3) and a new genotype, *T. orientalis* type N4 in *R. microplus* infesting cattle. The result of the study, highlight the genetic diversity of *T.orientalis* in Kerala and potential implications for regular mapping of genotypes in varying tick species in the region.

Conclusion

This study highlights the prevalence and distribution of tick species infesting cattle across different regions of Kerala, with *H. bispinosa* and *R. annulatus* being the most common. The detection of *T. orientalis* in *R. annulatus* ticks, particularly the Buffeli and Buffeli/Chitose mixed genotypes, confirms the potential role of this tick species as a vector for oriental theileriosis in Kerala. Phylogenetic analysis demonstrated a close relationship between the local strains and the pathogenic Ikeda strain, suggesting the presence of virulent forms of *T. orientalis* in the region. The findings emphasize the urgent need for targeted tick control measures and continuous surveillance to reduce the risk of tick-borne diseases in cattle, safeguarding livestock health and mitigating economic losses in tropical climates like Kerala. Further research is warranted to explore additional genotypes and transmission dynamics, contributing to more effective control strategies.

Acknowledgements

The authors are thankful to the Dean, College of Veterinary and Animal Sciences, Mannuthy for providing the facilities necessary to carry out the study.

Conflicts of interest

The authors declare that they have no conflict of interest.

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