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Molecular Confirmation of *Rhipicephalus haemaphysaloides* Infesting Ruminants in Wayanad, Kerala, India

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A B S T R A C T

Rhipicephalus haemaphysaloides is a prevalent multi host tick species with high vector potential in south India. There are several reports based on morphological identification of the tick species from various parts of the country. However, there were limited attempts for molecular confirmation of this tick species. In the present study, the presence of *R. haemaphysaloides* in Wayanad, Kerala was confirmed by polymerase chain reaction based on amplification of mitochondrial 16S rRNA gene and V4 region of 18S rRNA gene. Two tick samples, one engorged on a bovine male calf of Pookode, Wayanad and other engorged on a sambar deer in Meppadi were used for the study. Phylogeny based on 16S rRNA and 18S rRNA of *R. haemaphysaloides* revealed genetic relatedness with Chinese isolates of the tick species.

Introduction

Parasitic diseases are one of the major obstacles for the health of the animals, thereby causing severe economic constraints globally Ticks are the obligate haematophagousecto parasites of both livestock and humans. They were rank second after mosquitoes as vectors of human diseases. Ticks play an important role in the transmission of various pathogens like bacteria, viruses, protozoa and helminths, many of which have zoonotic significance (de La Fuente *et al.*, 2008). Tick infestation results in direct damages like reduction in milk yield and live weight and indirect effects due to pathogen transmission and toxicosis resulting in paralysis, irritation and allergy (Aktas, 2014).

The direct injuries due to ixodid ticks are severe in tropical climate (Ghosh and Nagar, 2014). There is a recent surge in studies regarding tick and tick borne diseases due to

the changes in tick distribution mainly contributed by the changes in climate (Jaenson*et al.,* 2009). According to Burger *et al.,* (2014), 904 tick species were identified globally. Almost 80 per cent of the tick species comes under the family Ixodidae or hard ticks (705 species). Family Argasidae or soft ticks contain 198 species and one under the family Nuttalliellidae.

India is home for approximately 109 tick species, under 12 genera (Ghosh *et al.,* 2007).The climatic conditions in India are highly favorable for the propagation of ticks. Most predominant tick species identified in India belong to the genus *Rhipicephalus* and *Hyalomma* (Ghosh *et al.,* 2007).

The prevalence of *Rhipicephalus haemaphysaloides* based on morphological identification was recorded from various parts of India including Kerala (Geevarghese and Dhanda, 1995; Rajendran and Hafeez, 2003; Prakasan and Ramani, 2007; Soundararajan *et al.,* 2014). However, they were no studies regarding the molecular confirmation of the tick species. In the present study, molecular confirmation of *R. haemaphysaloides* was performed using ticks collected from Wayanad, a northern district of Kerala.

Materials and Methods

Tick samples were collected froma bovine male Calffrom College of Veterinary and Animal Sciences, Pookode, Wayanad. Another isolate was collected from a sambar deer from Meppadi, Wayanad. Ticks were morphologically identified (Arthur, 1960) and stored in -80°C until RNA extractionwas performed. RNA extraction was done using RNeasy Mini Kit (Qiagen, Netherlands) based on the manufacture's protocols. Synthesis of cDNA was performed by Revert Aid H minus First strand cDNA synthesis kit (Thermo Scientific, USA). This cDNA was used as template for the polymerase chain reaction (PCR). Molecular confirmation of tick species was done by PCR amplification of mitochondrial 16S rRNA gene and V4 region of 18S rRNA gene (Crampton *et al.,* 1996; Kumar *et al.,* 2011). The primer sequences for PCR amplification are mentioned in table 1.

Amplified PCR products were analyzed by gel electrophoresis using 1.5 per cent agarose gel. PCR amplified products were sequenced at Sci. Genome Pvt. Ltd., Cochin and the sequence data were BLAST analyzed.

Phylogenetic analysis was performed for both 16S rRNA and 18S rRNA genesby Neighbor-joining tree method using Mega 7. Nucleotide sequences were aligned by Clustal W for analysis. Akaike information criterion implemented in MEGA 7.0 was used to determine the best fitting models. The evolutionary distances were computed using Tamura 3-parameter method and Jukes-Cantor method for 16S rRNA and 18S rRNA respectively.

Results and Discussion

Tick isolates from Pookode and Meppadi were morphologically identified as *R. haemaphysaloides.* Molecular analysis of tick mitochondrial16S rRNA gene of Pookode and Meppadi isolates revealed 95 per cent and 93 per cent identity respectively with *R. haemaphysaloides* (AY972534) from China.

BLAST analysis of 18S rRNA sequence of Pookode and Meppadi isolate showed 96 per cent and 100 per cent identity respectively with *R. haemaphysaloides* (DQ839552) from China. The sequences were submitted to Gen Bankto obtain accession numbers. Pookode isolate was assigned with accession numbers, KU895511 and KU895510 for 16S rRNA and 18S rRNA respectively. Similarly, Meppadi isolate was assigned with accession numbers

MF351994 and MF351848 for 16S rRNA and 18S rRNA respectively.

Phylogenetic analysis of mitochondrial16S rRNA revealed that both Pookode and Meppadi isolates of ticks were genetically closely related. Also, both shared identity with Chinese and Thailand isolates of *R. haemaphysaloides.* Based on evolutionary analysis of 18S rRNA, Pookode isolate of *R. haemaphysaloides* was more ancestral to both Meppadi and Chinese isolates (Figs. 1 and 2).

In the present study, the presence of *R. haemaphysaloides* in Wayanad was confirmed by molecular techniques. *R. haemaphysaloides*is usually prevalent in Oriental, Australasian and Palearctic zoogeographic regions with wide host range. The usual hosts of these ticks are birds and mammals including humans (Guglielmone *et al.*, 2014). *R. haemaphysaloides* is one of the most abundant cattle ticks in Sri Lanka (Diyes and Rajakaruna, 2015). Rajendran and Hafeez (2003) reported a prevalence of 3.29 per cent for *R. haemaphysaloides* among crossbred cattle of Andhra Pradesh. Soundararajan *et al.*, (2014) recorded a prevalence of 3.13 per cent in goats of Tamil Nadu. Prakasan and Ramani (2007) reported *R. haemaphysaloides* from cattle, buffalo, goat and pigs in Kerala.

Fig.1 Molecular phylogeny based on 16S r RNA. The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.56035004 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Tamura 3-parameter method and are in the units of the number of base substitutions per site. The rate variation among sites was modeled with a gamma distribution (shape parameter = 1). The analysis involved 14 nucleotide sequences

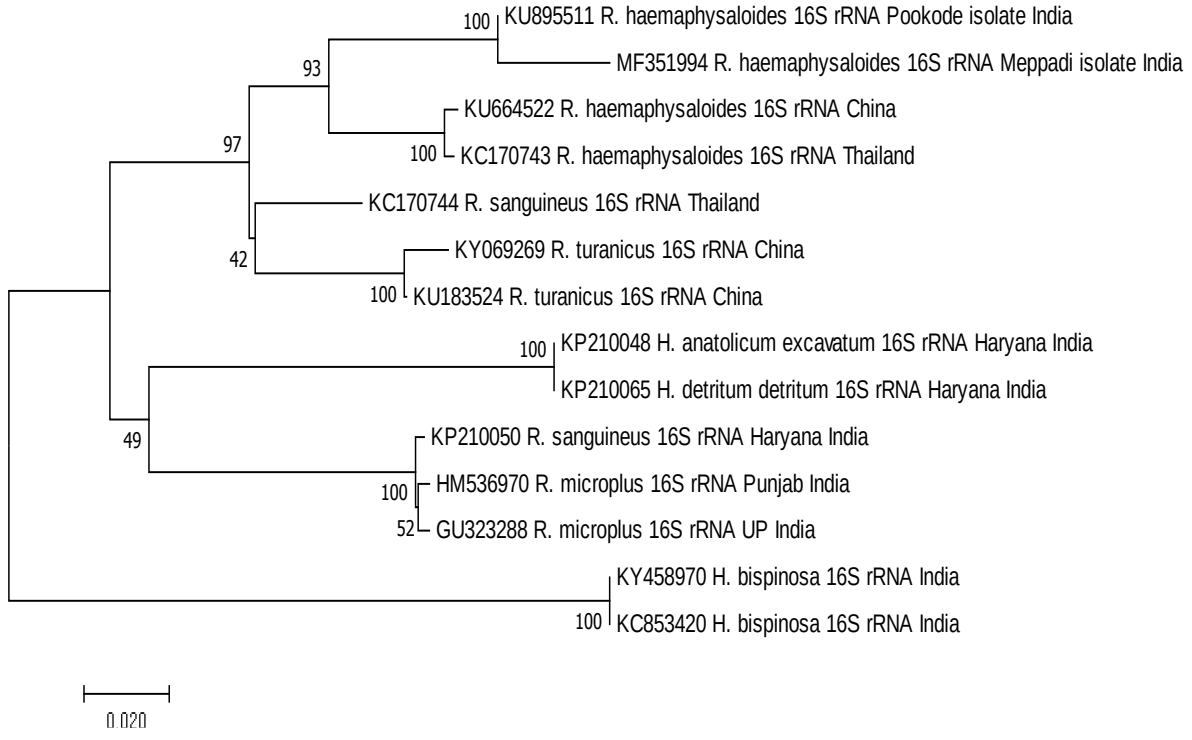

Fig.2 Molecular phylogeny based on 18S rRNA. The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 1.67598566 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Jukes-Cantor method and are in the units of the number of base substitutions per site. The analysis involved 11 nucleotide sequences

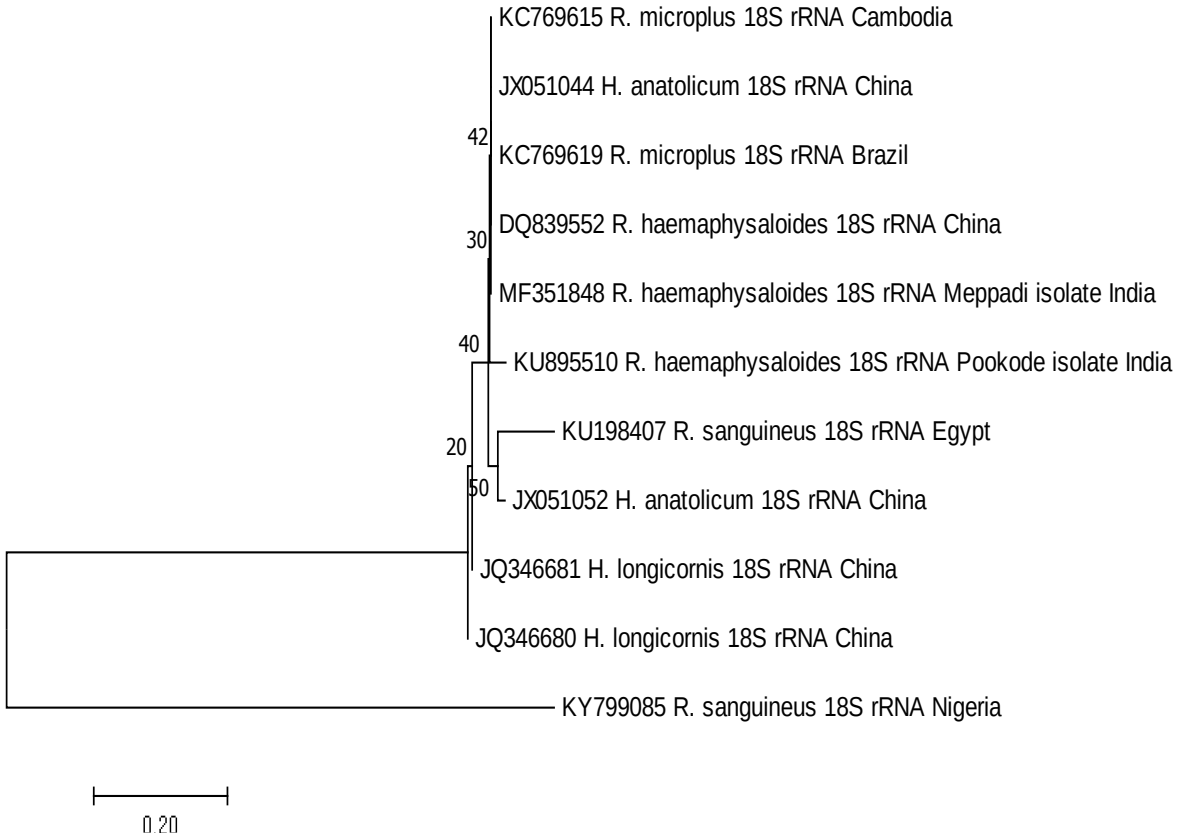

Table.1 Primers used for amplification of 16S r RNA and 18 S rRNA from ticks

Sl. No.	Gene	Primers	Amplicon length	Reference
Ticks	16S ribosomal RNA gene TIR	Forward-5' CCGGTCTGAACTCAGATCAAGT 3' Reverse- 5' GCTCAATGATTTTTTAAATTGCTG3'	450bp	Kumar *et al.*, (2011)
Ticks	V4 region of 18S ribosomal RNA gene TV4	Forward- 5' GGAGGGCAAGTCTGGTGC 3' Reverse- 5' CCATACAAATGCCCCCGTCTG 3'	338bp	Crampon *et al.*, (1996)

R. haemaphysaloides is having both medical and veterinary significance as they can act as vector for various pathogens. Laboratory studies revealed their role in the transmission of *Rickettsia* (Hsu *et al.,* 2011). Tsui *et al.,* (2007) detected spotted fever group rickettsial organism in *R. haemaphysaloides* using molecular methods in Taiwan. Experimental transtadial transmission of *Babesia microti* through *R. haemaphysaloides* in southern China was also reported (Li *et al.,* 2016). Bhat *et al.,* (1978) confirmed the role of *R. haemaphysaloides* for the transmission of Kyasanur forest disease (KFD). Thus, the presence of *R. haemaphysaloides* in Wayanad and its wide vector potential indicated the increased risk for both animals and humans in the area.

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