

Analysis on the Molecular Detection of *Rickettsia felis* in *Rhiphicephalus sanguineus* Ticks Found in India

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ABSTRACT

Tick borne diseases (TBDs) have remained a major concern throughout the world. The cases of TBDs have increased in past few years. Moreover, new emerging pathogens are being detected from ticks which have led the scientists to reveal more on this field. Among various pathogens transmitted from ticks, Spotted Fever Group rickettsia (SFGR) represents the huge number of new and emerging infectious pathogens but unfortunately has been neglected. Therefore, the study was carried out to detect SFGR from ticks collected from Gwalior. This report is a description *Rickettsia felis* which is a potential human pathogen detected in adult *Rhiphicephalus sanguineus* tick collected from dogs in Gwalior, India. For SFGR screening in the ticks, partial conserved region of Citrate Synthase (*gltA*) and Outer Membrane Protein B (rOmpB) genes were targeted. Both of the genes were amplified and thus indicated the presence of the rickettsial pathogen and its associated risk. Five percent (5.19%) of the screened ticks were PCR positive for SFGR. PCR amplicons were sequenced further. Additionally, Immunofluorescence assay (IFA) test was also done to double sure the result obtained. The sequencing result of *gltA* gene showed 97.87 percent similarity to *R. felis* (MH194353) and for rOmpB gene there was 99.47 percent similarity to *R. felis* (MG451836). This is the first report of *R. felis* DNA from *Rh. sanguineus* ticks in India. Further studies are required for clarity on the distribution of the *R. felis* in ticks and its transmission to human being. Thus, a comprehensive awareness is much needed for tackling rickettsial pathogen and subsequent steps leading to an improved health management in India.

KEY WORDS: RICKETTSIA FELIS. RHIPHICEPHALUS SANGUINEUS. CITRATE SYNTHASE GENE. OUTER MEMBRANE PROTEIN B. INDIA. TICKS.

INTRODUCTION

Since 20th century, ticks were known to transmit human infectious diseases transmitting variety of pathogens (Parola and Raoult, 2001). Novel SFGR species have been detected from different tick species around the

globe (Noh et al., 2017; Rivera-Páez et al., 2018). Many researchers have conducted tick prevalence in different areas. Recently surveillance was carried out in India on the distribution of tick species to have insight on its vector ecology. *Rh. sanguineus*, also known as the brown dog tick are prevalent all over the world. They mostly parasitize dogs, but feeding on humans have also been reported (Palmas et al., 2001; Dantas-Torres, 2007; Ghosh et al., 2020).

Numerous pathogens such as *Babesia canis*, *Ehrlichia canis*, *R. rickettsii*, *R. conorii* are known to be transmitted by *Rh. sanguineus* causing diseases in humans as well as in animals (Brumpt et al., 1932; Parker et al., 1933; Groves et al., 1975). For better knowledge on SFGR and its diagnosis, much research is needed in Asia (Robinson et al., 2019). There are abundant literatures prevailing in

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the last decade which reported several native and new species of *Rickettsia* detected from ticks (Shao et al., 2020; Binetruy et al., 2020).

In spite of these studies, we lack in the complete understanding of SFGR interaction with ticks for its transmission and its impact on Human as well (Tomassone et al., 2018). Keeping in view the role of ticks in passive detection of *Rickettsia*, we have conducted the study to detect the presence of SFGR in *Rh. sanguineus*, a potential vector for *Rickettsia* in and around Gwalior of India.

MATERIAL AND METHODS

Ticks were collected from dogs in Tekanpur location of Gwalior shown in figure 1. with geographical coordinates of 26° 22' 0" North, 78° 18' 0" East. Collected ticks were kept in vials with proper label and transported. Live unengorged ticks were sorted and were washed with 70% ethanol followed by distilled water, dried and viewed under Leica EZ4D and Leica M205A stereo zoom microscope attached with DFC500 camera. Ticks were morphologically identified as *Rh. sanguineus* using taxonomical keys which involved broader Capitulum, comma-shaped and elongated spiracles throughout, short palps (Walker et al., 2005). Identified ticks were preserved in 90% ethanol in -200C till use for DNA extraction. Each tick pool used for DNA extraction comprise of 2 ticks. DNA extraction was performed using DNeasy blood and tissue kit (Qiagen) according to the manufacturer's instruction (Walker et al., 2005).

For qualitative analysis, the extracted DNA was checked by running it on 1% agarose gel electrophoresis. To corroborate the result of morphological identification, 16SrRNA and COI gene of tick was amplified using the primer and protocol of previously published literatures (Black et al., 1994). The sequences amplified were submitted to NCBI. Subsequently screening of *Rh. sanguineus* tick was done for *Rickettsial* pathogen. Partial regions of *gltA* and *rOmpB* genes (nested PCR) of SFG *Rickettsia* were amplified using primers and protocols generating product of 401bp; 511bp and 420bp respectively (Chitimia et al., 2010).

Rickettsia conorii DNA (Vircell) was taken as positive control whereas nuclease free distilled water as negative control (Labruna et al., 2004; Choi et al., 2005). The positive PCR amplicons were subsequently excised and purified using Qiagen gel extraction kit. Further characterization of the PCR products was done by Sanger's sequencing (Genotypic technology, Bangalore). The recovered nucleotide sequences were compared with the available sequences in Genbank using BLAST and phylogenetic tree was made for evolutionary analysis of the SFGR detected. Furthermore, IFA test was done to support and corroborate the result obtained from PCR reaction. For IFA, a smear was made from triturated tick (pool of 2 ticks) and subsequently air dried (Choi et al., 2005).

The slides were fixed in Acetone at 4°C for 20 minutes followed by washing step (3 times) in PBS. Thereafter, *Rickettsia conorii* Antibody (1: 128) was added over the smear made and incubated for 30 minutes at 37°C in humidity chamber. Slides were washed with PBS followed by an addition of a drop of FITC conjugated secondary antibody and further incubated in humidity chamber for 30 minutes at 37°C in dark. Afterwards the slides were washed in PBS. Two drops of mounting media were added to each slide and coverslip was placed removing any air bubbles. The slides were observed at 400X magnification in fluorescence microscope (Labruna et al., 2004; Choi et al., 2005).

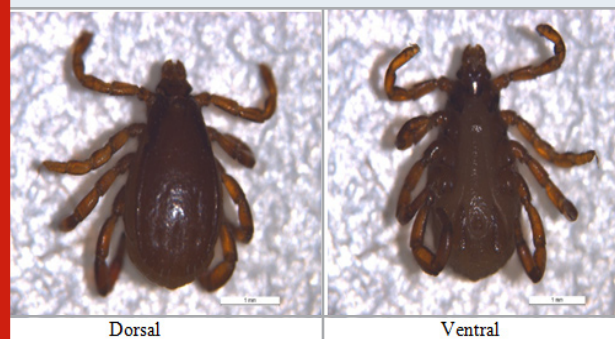
Figure 1: Study Area



RESULTS AND DISCUSSION

All ticks collected from dogs were morphologically identified as *Rh. sanguineus* (Fig: 2).

Figure 2: *Rhiphicephalus sanguineus* (male)



The taxonomical keys used involved broader Capitulum, 'Simus' pattern of punctuation, elongated spiracles throughout, short palps (Walker et al., 2005). Sequences for 16SrRNA and COI gene of ticks were successfully amplified and were submitted in NCBI with accession number MH765330 and MH765337 respectively. A total 77 tick DNA pool were used for screening of SFGR. The PCR result showed that, out of 77 tick pools screened, four pools were found positive for SFGR DNA with 5.19% positivity in ticks by both the genes (figure 3) (Walker et al., 2005).

PCR positive amplicons were sequenced. The sequencing result for *gltA* and *rOmpB* genes showed 97.87 percent

identity to *R. felis* (MH194353) from Brazil and 99.47 percent identity with *R. felis* (MG451836) from Italy. The phylogenetic tree was made using maximum likelihood and Tamura-Nei model under 1000 bootstrap replicates in Mega X (figure 4 & 5) (Walker et al., 2005).

Figure 3: Agarose gel electrophoresis of *gltA* and *rOmpB* gene PCR from screened tick species

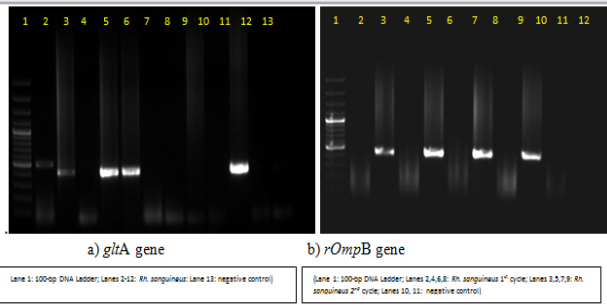
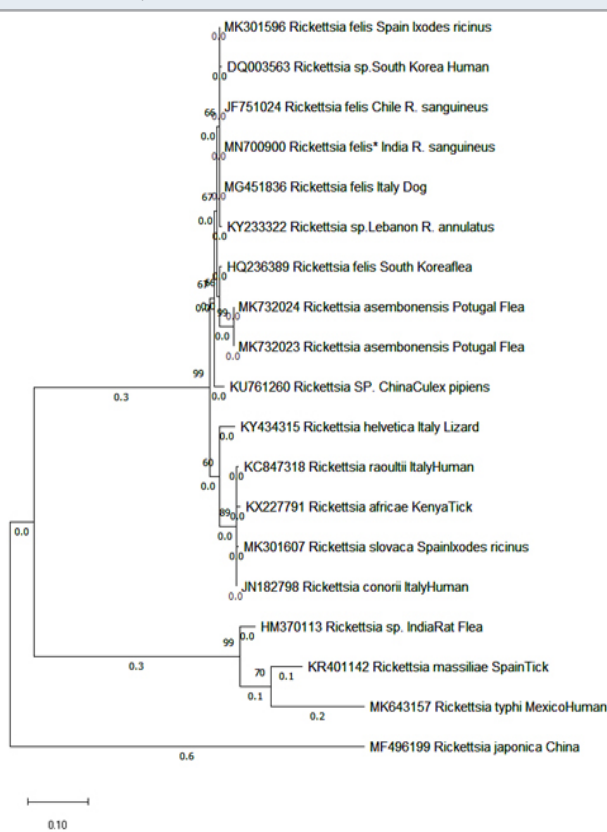


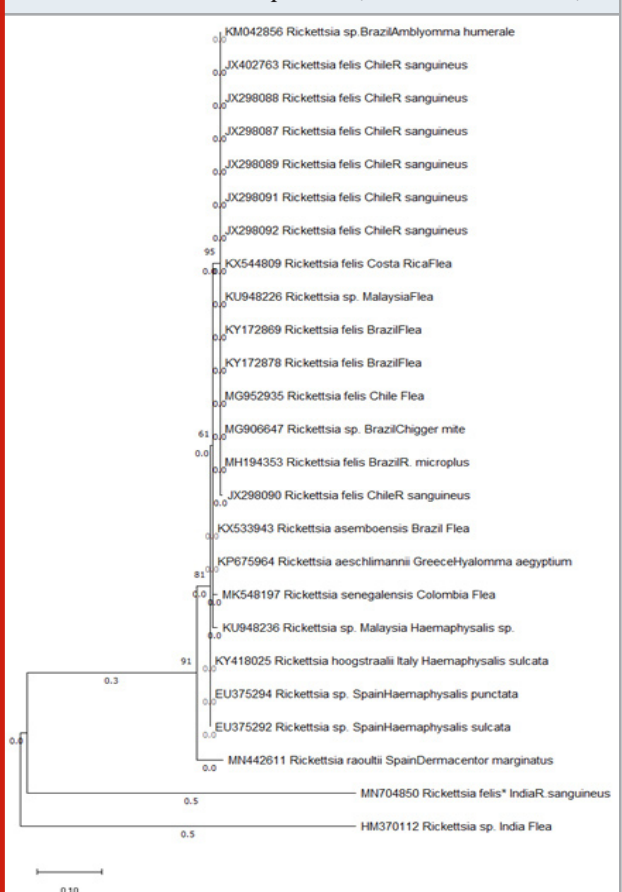
Figure 4: Maximum likelihood (ML) tree inferred from *rOmpB* gene partial sequences of *R. felis* detected in the present study (*) and sequences from GenBank using MEGA X. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site (next to the branches)



The gene sequences for *R. felis* were deposited in GenBank under accession number MN704850 for *gltA* gene and MN700900 for *rOmpB* gene. In case of IFA, 25 pools of ticks were screened, out of which 4 tick pools showed positive result (figure 6) (Walker et al., 2005).

R. felis has been proposed as a new member for the spotted fever group (SFG) of the genus *Rickettsia*. First human pathogenicity linked with *R. felis* was from Texas in 1994 (Schriefer et al., 1994; Bouyer et al., 2001). It is widely distributed all over the world and has become a major threat for human with its flexibility of adapting a broad array of ectoparasites and mammals including humans as well (Pérez-Osorio et al., 2008). In recent time *R. felis* was also reported from various mosquito species and was proposed experimentally that *Anopheles gambiae* can act as possible vector of *R. felis* (Dieme et al., 2015). Likewise, the spreading of chikungunya virus infection from the Indian Ocean to parts of world, Zika virus from French Polynesia to America (Parola et al., 2016).

Figure 5: Maximum likelihood (ML) tree inferred from *gltA* gene partial sequences of *R. felis* detected in the present study (*) and sequences from GenBank using MEGA X. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site (next to the branches)

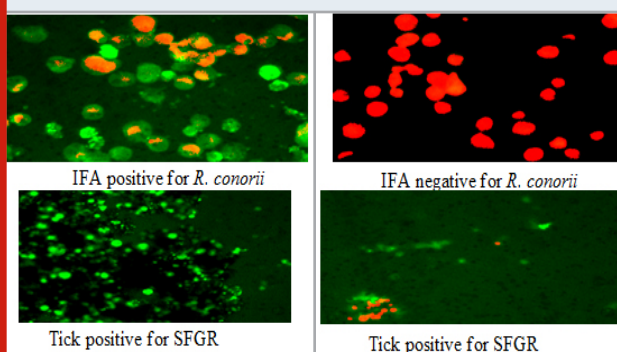


Parola et al., (2016) raised concern on the possibility of *R. felis* outbreak from mosquitoes also. Although *R. felis* has been detected in mainly in cat flea, it has also been documented from several ticks, including *Rh. sanguineus* (Cardoso et al., 2006; Abarca et al., 2013). About thirty-nine species of ectoparasites have been linked with *R. felis* which includes numerous species of fleas, ticks, lice, and mosquitoes whereas Oliveira et al. (2008)

confirmed that *R. felis* is circulated and maintained in different ectoparasites including *Rh. sanguineus* as well. *Rh. sanguineus* tick from China was also found to be positive with *R. felis* with a positivity rate of ten percent, whereas in our study it was five percent (Zhang et al., 2014; Brown et al., 2016).

Additionally, *R. felis* was detected from *C. felis*, *Rh. sanguineus* and *Dermacentor nitens* with a varying positivity rate of 22.22%, 3.22% and 1.75% respectively (Oliveira et al., 2020). These variations in the positivity rate of *R. felis* are affected by various factors such as using different methodologies used, the environmental condition of the vectors and the presence of *Rickettsia* reservoirs (Khrouf et al., 2014). *Rh. sanguineus* is the known vector of several pathogenic agents like *Ehrlichia canis*, *Coxiella burnetii*, *R. rickettsii*, and *R. conorii* which have a great zoonotic importance (Dantas-Torres et al., 2008). In India, positives cases for *R. felis* were reported in 11 patients from south eastern India whereas, flea borne rickettsia was also detected from cat flea *Ceratophyllus fasciatus* from Western Himalayan region, however its prevalence in arthropod vector was not studied (Chahota et al., 2015; Koralur et al., 2016).

Figure 6: Immunofluorescence (IFA) of ticks



In similar line, *R. felis*-like organism (*Rickettsia* sp. genotype RF2125) was reported from Delhi, Mumbai and Rajasthan of India and its high prevalence in flea associated with dogs as reservoir was implicated as important zoonotic threat (Hii et al., 2015). To support our result obtained from PCR, we have done IFA which is a known confirmatory technique as well. Because of greater sensitivity and specificity, PCR based method is preferred over direct detection method such as IFA (Massung et al., 1998). IFA results in existing study exhibited 16% tick positivity to SFGR. This confirms the existence of SFGR in *Rh. sanguineus* ticks from Gwalior. Thus, current study indicates that *Rickettsial* infections are circulated and maintained in ticks but are underrated and its real existence on public health is left unknown. Our result showed that *R. felis* is circulated in the ticks of Gwalior and it therefore becomes imperative to elucidate the status of SFGR in ticks associated with dog, mainly in *Rh. sanguineus* (Koralur et al., 2016; Oliveira et al., 2020).

Additionally, so far there is no published report of *R. felis* DNA detected either from patient or from any of tick vector in India. Our study is the first report of *R. felis* DNA from *Rh. sanguineus* ticks in India. Since *Ctenocephalides felis* is the only known biological vector for *R. felis*, the report of *R. felis* DNA in tick raises many questions about the role of *Rh. sanguineus* as vector and also the role of dogs as reservoir. *R. felis* has been documented to be transmitted through co-feeding of numerous arthropods, horizontally from infected to uninfected Arthropods, via direct contact or through the consumption of contaminated blood meals and also via mating, however its transmission route in India is not systematically studied. Although vector status of *Rh. sanguineus* in India is not established for *R. felis*, the biological risk associated with *R. felis* could not be neglected. Taking all these facts into consideration, our study emphasizes the need of extensive research on association of *R. felis* with *Rh. sanguineus* ticks of India which may act as a potential vector and transmit this human pathogen thereby becoming a global threat (Nguyen et al., 2020).

CONCLUSION

This is the first report of presence of *R. felis* DNA in *Rh. sanguineus* ticks from Gwalior, India. There is a huge scarcity of information regarding TBDs in India, therefore these studies will help to elucidate and resolve the unsolved queries regarding TBDs. The result of the study also emphasizes on the presence of novel *Rickettsia* species in India which still needs to be explored from different tick vectors. Further studies are required to have more clarity on the distribution of the *R. felis* in ticks and its transmission to human being. These kinds of studies are required to fill the wide gaps in the broad areas of ticks and tick-borne diseases.

Conflict of Interest: There were no conflict of interest among the authors.

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Author's contribution: All the authors have contributed to the design and conceptualization of the study. The conduct of experiment and manuscript writing was done by Pooja Ghosh. The morphological identification and the idea behind the study were done by Sachin Tikar. Data analysis was done by Mahendra K. Gupta. Sample collection was done by Abhay D. Gupta and D. Sukumaran. All the authors have read and approved the final manuscript.

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