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Article

The bacterial microbiome of *Rhipicephalus* (*Boophilus*) *annulatus* (Acari: Ixodidae)

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ABSTRACT
Despite the role of ticks in many infectious diseases, studies on the diversity of bacteria in tick species are limited. There have not been any scientific articles available that explore the bacterial microbiota associated with *Rhipicephalus* (*Boophilus*) *annulatus* (Say) ticks in India. In this study, we attempted to gather elaborate data on the bacterial fauna associated with *R. annulatus* through conventional culturable and culture-independent (Next Generation Sequencing —NGS) methods. Two locations of zoonotic significance within the Western Ghats region were selected. Tick samples were obtained from grazing cows (*Bos indicus*) from June 2023 to September 2023. The conventional culturable method revealed three phyla and the NGS exposed 19 phyla. The culturable method reported Actinobacteria as the abundant phyla (57.14%), followed by Proteobacteria (28.57%) and Firmicutes (14.28%). According to the NGS data, phylum Firmicutes (35.81%) was the most abundant phylum with differences at 95% confidence intervals. The study obtained a total of eight bacterial genera of pathogenic potential. Given the medical importance of the identified bacterial strains in Healthcare-Associated Infections (HAI), this study highlights the need for further research on tick-associated microbiomes.

KEYWORDS: Culturable method, India, Kerala, Next Generation Sequencing, tick.

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INTRODUCTION

Ticks are ectoparasites of vertebrates whose larval, nymphal, and adult stages depend on hematophagy. Ticks are known to have considerable medical and veterinary importance as they transmit bacterial, viral, and protozoal pathogens through their saliva. Ticks harbour numerous microorganisms, including pathogens, symbionts, and commensals (Parola and Raoult 2001; Jongejan and Uilenberg 2004). The rural agroecosystems of forest fringes can sustain many pests and parasites along with their hosts. The man-wild conflict and global environmental changes make the forest fringes more susceptible to various diseases, such as vector-borne zoonoses (Bouchard *et al.* 2019). Previously, the studies on tick microbiome were mainly focused on a few specific pathogenic

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bacteria, but the exploration of bacterial diversity is accelerated nowadays with modern approaches like Next Generation Sequencing (NGS) (Chaorattanakawee *et al*. 2022). As the number of bacteria cultivable by the classical culturable method is very small, new approaches like NGS can be applied for the exploration of microbes associated with each tick (Greay *et al.* 2018). Even after 13 years of pioneering work, bacterial diversity studies among different Indian tick species using NGS methods remain limited (Andreotti *et al*. 2011). This research employs both conventional culturing and NGS methods to address this gap.

The *Rhipicephalus*, also known as brown ticks, is a genus with a hexagonal basis capituli. Its immature stages are difficult to identify morphologically. *Rhipicephalus* (*Boophilus*) *annulatus* (Say) was included under the genus *Boophilus*, but recent molecular evidence suggests it belongs to the genus *Rhipicephalus* and subgenus *Boophilus* (Amrutha *et al*. 2023). It is a widely distributed one-host tick and one of the main vectors that transmit diseases such as bovine babesiosis and anaplasmosis. Even though *R. annulatus* is called the cattle tick, it can infest wild animals and humans as well (Walker *et al*. 2000; Scoles *et al*. 2007; Adham *et al*. 2009; D'Amico *et al*. 2017).

Anthropogenic interventions in the forest fringes of the Western Ghats have been recognised as a primary cause of endemic zoonotic outbreaks such as Nipah fever, spotted fever, and monkey fever. The recent emergence of these diseases in the Kozhikode and Wayanad districts of Kerala has raised public health concerns (Walsh *et al.* 2019; Pallivalappil *et al.* 2020). Frequent monitoring and control of vectors in these locations can prevent vector-borne disease transmission to some extent. This study aims to provide a comprehensive diversity analysis for culturable and unculturable microbiomes associated with *R. annulatus* ticks, collected from the grazing cattle of peripheral forest areas of the Western Ghats, Kerala, India.

MATERIALS AND METHODS

Collection and identification of ticks

The study was conducted from June 2023 to September 2023 in the forest fringes of Perambra area (11° 35' 17.6" N, 75° 51' 56.3" E) of Kozhikode district and Pulpally (11° 47′ 34.8″ N, 76° 9′ 54″ E) area of Wayanad district in Kerala, India. Ticks from 10 grazing cows (*Bos indicus*) were collected and preserved for microbiological analysis. Morphological keys were used to identify tick species, while confirmation was achieved by molecular characterisation (Geevarghese and Mishra 2011; Kazim *et al*. 2022). The ticks were kept alive for further analysis.

DNA amplification by polymerase chain reaction (PCR)

The tick DNA was isolated using the NucleoSpin® Tissue Kit (Macherey-Nagel) following the manufacturer's instructions. After checking the quality of the DNA using agarose gel electrophoresis, the DNA isolates were subjected to PCR using the *COX1* primer designed by Folmer *et al*. (1994) (forward: 5' GGTCAACAAATCATAAAGATATTGG 3'; reverse: 5' TAAACTTCAGGGTGACC AAAAAAT CA 3'). The PCR amplification was carried out in the GeneAmp PCR System 9700, Applied Biosystems. The PCR profile of *COX1* gene is given in Table 1. The sequence quality was analysed using Sequence Scanner Software v1 (Applied Biosystems) (Anoopkumar *et al*. 2019). The DNA sequences obtained were compared with the sequences in the NCBI database using the nucleotide BLAST tool and submitted to the GenBank. From the identified ticks, *R. annulatus* ticks were selected for microbial exploration as the species was not considered previously for bacterial faunal analysis.

Cultivation, isolation, and identification of culturable bacteria

To isolate bacterial fauna associated with *R. annulatus*, 10 ticks were surface sterilised using absolute alcohol to discard the bacteria on their body surface. The ticks were crushed and transferred to an Eppendorf tube to create a master solution. A serial dilution of the solution was carried out, with

varying ratios. The dilutions were spread into Petri plates containing different bacterial culture media (nutrient agar, blood agar, and MacConkey agar). The cultures were incubated at 29 °C for 72 hours, observed every 24 hours. Individual colonies were picked from the plates to create distinct bacterial subcultures. After 24 hours of growth, isolated bacterial subcultures were taken for DNA isolation using the Origin-Genomic DNA Kit ODP304. Bacterial *16S rRNA* gene amplification of DNA isolate of respective bacterial cell culture was performed using a TAKARA PCR thermal cycler (primers used were forward: 5' AGAGTTTGATCCTGGCTCAG 3'; reverse: 5' GGTTACCTTGTTACGACT T 3') (Monciardini *et al*. 2002). The PCR profile of *16S rRNA* gene is given in Table 1. The amplified PCR product was further sequenced by following Sanger's dideoxy chain termination sequencing method (Sanger and Coulson 1975). The sequences were compared with NCBI database sequences using nucleotide BLAST and deposited in GenBank to obtain accession numbers.

Table 1. PCR amplification profile of Tick and Bacterial DNA.

PCR Step	Amplification of Tick DNA		Amplification of Bacterial DNA	
	Time (s)	Temperature (°C)	Time (s)	Temperature (°C)
Initial Denaturation	30	98	300	95
Denaturation	5	98	10	95
Annealing	10	45	60	50
Extension	15	72	45	72
Final Extension	60	72	240	72

Bacterial faunal diversity analysis by *Next Generation Sequencing method*

The second pool of 10 surface-sterilised *R. annulatus* ticks was subjected to NGS. The metagenomic DNA was extracted using DNA extraction kits (DNeasy Tissue Kit, Qiagen). The purity of the extracted metagenomic DNA sample was analysed using NanoDrop (Thermo Fisher Scientific, USA) by determining the A260/280 ratio. The primers targeting the *16S V3-V4* region of the *16S rRNA* gene were used to set up the primary amplicon (forward: 5' GCCTACGGGNGGCWGCAG 3'; reverse: 5' ACTACHVGGGTATCTAATCC 3') (Bisht *et al*. 2018). After the qualification using nanodrops, the sample was processed for library preparation (Nextera XT library preparation kit). It was then sequenced with paired-end 2×300 reads on the MiSeq system (Illumina, USA) (Edgar 2010; Teng *et al.* 2018). Bioinformatic analysis of the microbial community and data visualisation were done through Quantitative Insights into Microbial Ecology (QIIME) software (Caporaso *et al.* 2010). Before processing the data of the sample, Trimmomatic V0.38 was used to remove adapter sequences, reads with unknown nucleotides "N" larger than 5% (ambiguous reads), and reads with more than 10% quality threshold (QV) < 25 phred scores (low-quality sequences), along with a sliding window of 20 bp and a minimum length of 100 bp. Paired-end data were stitched into single-end reads using FLASH (V1.2.11). High-quality clean reads were then denoised, and chimeric sequences were filtered through DADA2. The taxonomic classification of amplicon sequence variants was performed with the q2-feature classifier using a pre-trained classifier based on the SILVA database, followed by the calculation of diversity metrics within the sample (α-diversity; Shannon's index) (Bolger *et al*. 2014).

RESULTS

Collection and identification of ticks

A total of 126 ticks were collected from 12 adult cows (*Bos indicus*). Out of 20 cows examined, 60% of them were found to be infested with ticks. The tick infestation rate of domestic cows of Pulppally (58%) was higher than that of Perambra (42%). Three species of ticks, belonging to two

genera, were obtained. The genus *Rhipicephalus* dominated, though there were two species*: R. microplus* (Canestrini) (33.4%) and *R. annulatus* (19%). *Haemaphysalis bispinosa* (Neumann), of the genus *Haemaphysalis*, represented 47.6% of the total tick collection. The molecular sequencing using *COX1* gene confirmed the identity of two closely related ticks as *R. microplus* (NCBI accession number: OR250142) and *R. annulatus* (NCBI accession number: OR230585).

Bacterial faunal diversity by culturing method

A total of six morphologically distinct bacterial colonies were isolated from *R. annulatus* ticks. They were identified up to the genus level, through molecular characterisation using the *16S rRNA*. Out of six bacterial strains obtained from the conventional cultural method, four were gram-positive, viz., *Micrococcus* sp. (NCBI accession number: PP716825.1)*, Microbacterium* sp. (NCBI accession number: PP724712.1), *Corynebacterium* sp. (NCBI accession number: PP724413.1), and *Bacillus* sp. (NCBI accession number: PP716757.1). Two gram-negative strains identified were *Ralstonia* sp. (NCBI accession number: PP716819.1), and *Stenotrophomonas* sp. (NCBI accession number: PP716836.1). These six bacterial strains fall under six families (Micrococcaceae, Microbacteriaceae, Corynebacteriaceae, Bacillaceae, Burkholderiaceae and, Lysobacteraceae), four classes (Actinomycetes, Betaproteobacteria, Bacilli and Gammaproteobacteria), and three phyla. The phylum Actinobacteria had the highest abundance (57.14%), followed by Proteobacteria (28.57%) and Firmicutes (14.28%). Similarly, Actinobacteria has a larger diversity at the genus level than the other two phyla.

Bacterial faunal diversity analysis by *Next Generation Sequencing method*

The total DNA isolated from *R. annulatus* ticks were subjected to the DNA quality assessment using Nanodrop. This revealed a metagenomic concentration of 42.6 ng/µL, with a 260/280 ratio of 1.82 and a 260/230 ratio of 1.51, indicating suitable purity for downstream applications. After removing the adapter sequences, ambiguous reads, and low-quality sequences, a total of 6,22,40,300 high-quality (HQ) bases and 1,23,393 HQ reads were obtained. The HQ reads were subjected to operational taxonomic unit (OTU) identification at 97% sequence similarity and taxonomic assignment of OTUs using the Green genes database and the QIIME module (Bisht *et al.* 2018). The data generated during the study is deposited in the Sequence Read Archive (SRA) of the NCBI database under accession number PRJNA1129136. The method also revealed 19 bacterial phyla, including 19 families. The Krona-based diagram (Ondov *et al*. 2011) visualized that the phylum Firmicutes (35.81%) was the most abundant phylum with differences shown at 95% confidence intervals (Fig. 1). The class-level identification revealed the bacteria belonged to 19 classes, with the dominance of class Clostridia (25.69%). In this study, the genus and species-level abundance were not satisfactorily established, though the genus-level identification of *Prevotella* sp. belonging to the Prevotellaceae family was obtained. The alpha diversity indices for the NGS data, with a Shannon's index of 7.95 and a Simpson's index of 0.99, represent a highly diverse microhabitat.

DISCUSSION

Identifying the bacterial biome of tick species is very important, as all ticks are hematophagous arthropods and can transmit diseases to vertebrates (Bonnet *et al*. 2017). The exploration of the bacterial biome in *R. annulatus* through conventional culturable and NGS methods provided data on the abundance of bacteria, including genus-level identification of some opportunistic pathogens of public health importance. All six bacterial genera obtained were described as opportunistic pathogenic genera (Albertson *et al*. 1978; Alonso-Echanove *et al.* 2001; Laffineur *et al*. 2003; Tam *et al*. 2010; Bernard 2012; Brooke 2012; Yassin and Ahmad 2012; Ryan and Adley 2014; Siddiqui *et al*. 2022; Shi *et al*. 2023; Tokano *et al*. 2023). In this study, the order Clostridiales dominated with 23.78% abundance, and the most abundant family identified was Clostridiaceae (22.68%).

Unclassified genera from the family Clostridiaceae (9.75%) dominated at the genus level. *Clostridium sensu stricto* is a group of important human and animal pathogens. The pathogenic clostridial strains such as *C. botulinum* and *C. tetani* can produce neurotoxins, which are the causative agents of the disease's botulism and tetanus, respectively (Collins *et al*. 1994; Stackebrandt *et al*. 1999; Popoff and Bouvet 2013; Barash and Arnon 2014). The *Prevotella* sp. is a gram-negative, bacteria and the members of the genus *Prevotella* are commonly associated with the human oral cavity and guts (Ueki *et al*. 2007; The Human Microbiome Project Consortium 2012). The *Prevotella* colonization experiments conducted in mice revealed the capacity of these microbes to cause inflammatory responses. Many novel *Prevotella* species have been identified in the gastrointestinal and respiratory tracts, but their clinical significance is yet to be studied as they can cause pathological effects under certain circumstances (pathobionts) (Larsen 2017; Könönen and Gursoy 2021).

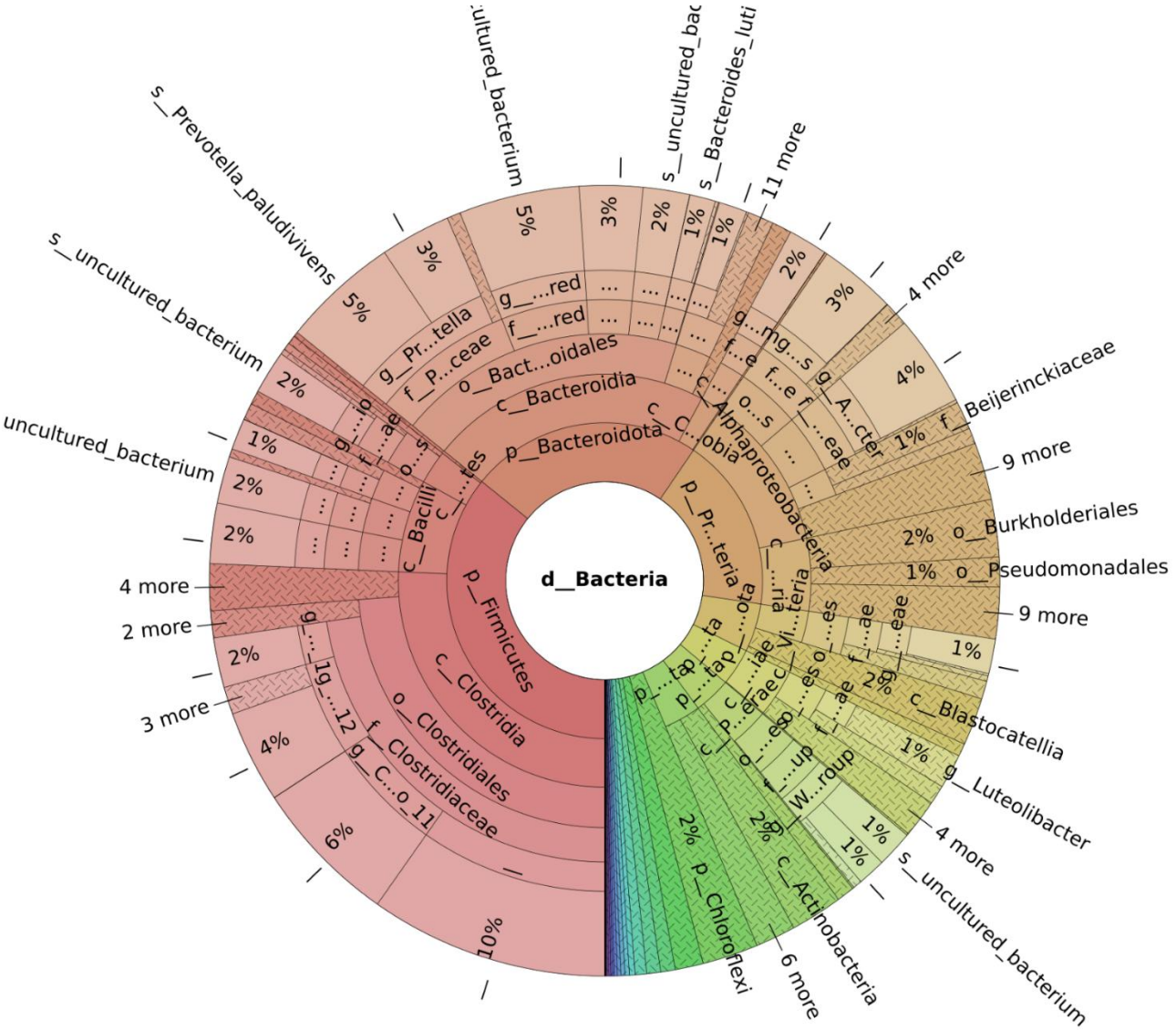

Figure 1. Krona chart showing relative abundance and diversity of NGS dataset.

In addition to the well-known pathogens that can trigger immunological reactions in domestic animals, pets, and humans, there is a plethora of bacteria that may or may not have pathogenicity (Yadav and Upadhyay 2021). Symbionts can interfere with pathogen transmission and their ability to colonise. The absence of certain bacterial strains in the microbiota of ticks cultured in sterile

environments can even alter the nature of the tick intestine (Narasimhan *et al*. 2014; Vayssier-Taussat *et al*. 2015). The opportunistic bacterial infections are a global burden of Healthcare-Associated Infections (HAI). The impact of HAI in immunocompromised people is higher than the general population. The multidrug resistance acquired by these bacteria is associated with high mortality in intensive care settings. In the light of increased importance of emerging tick-borne diseases, it is also important to study and monitor the bacterial fauna inhabiting the ticks to see if these bacteria can transmit through tick bites (Jernigan *et al*. 2020; Sikora and Zahra 2023).

Unfortunately, only a few publications are available regarding the bacterial diversity of ticks from India. This research gap includes studies involving different NGS techniques, such as pyrosequencing, nanopore sequencing, ion Torrent, etc. Most of the studies on microbial diversity associated with ticks were done using *16S rRNA* gene sequencing (Cabezas-Cruz *et al*. 2019). The pyrosequencing-based assessment of bacterial diversity in Turkish *R. annulatus* was revealed with a bacterial fauna dominated by the genus *Flavobacterium* and the bacterial strain *Stenotrophomonas maltophilia* also identified. In the present study, either the genus *Flavobacterium* or the bacteria, *S. maltophilia* were not obtained by NGS, but interestingly, *Stenotrophomonas* sp. was grown on various agar plates (MacConkey Agar, Nutrient Agar, and Blood Agar) (Tekin *et al*. 2017). This emphasises the role of geographical variation in tick microbiomes. The bacterial genera, *Micrococcus*, *Bacillus*, and *Ralstonia*, obtained through the culture method in this investigation were previously reported in *R. microplus* tick, through different metagenomic approaches (Xu *et al*. 2015; Molina-Garza and Galaviz-Silva 2020; Rojas-Jaimes *et al*. 2021). A member of the genus *Ralstonia*, *R. mannitolilytica* has been found in *R. sanguineus* ticks in Colombia by shotgun metagenomics (Paez-Triana *et al*. 2023). This bacterial strain was isolated also from the tick *Ixodes holocyclus* (Neumann) collected from Australia and was obtained by traditional microbial nutrient agar culturing (Murrell *et al*. 2003). A comparative analysis of cultivable bacteria isolates by MALDI-TOF mass spectrometry reported the presence of *Micrococcus* sp. and *Stenotrophomonas* sp. in tick species *Amblyomma cajennense* (Fabricius) and *Otobius megnini* (Dugès) (Molina-Garza and Galaviz-Silva 2020). The tick genera *Dermacentor*, *Haemaphysalis*, and *Hyalomma* were found to have *Microbacterium* sp., *Micrococcus* sp., and *Corynebacterium* sp. (Li *et al*. 2014; Song *et al*. 2021; Chigwada *et al*. 2022). Bacterial genera like *Rickettsia*, *Pseudomonas*, *Acinetobacter*, *Coxiella*, and *Flavobacterium* are often found in different tick genera, including *Ixodes*, *Rhipicephalus*, *Haemaphysalis*, *Dermacentor*, *Hyalomma*, and *Amblyomma* were not obtained in this study (Cabezas-Cruz *et al*. 2019). The well-known tick-borne pathogenic genera such as *Borrelia*, *Francisella*, *Anaplasma*, and *Ehrlichia* (Parola and Raoult 2001) were also not reported. The composition of the microbial population varies across different tick genera, and many other factors. The profile of tick microfauna is affected by host specificity, season, and geographical region, of which the tick samples were collected. The life stages of ticks and their feeding status are another important factor that affects the bacterial abundance pattern (Van Treuren *et al*. 2015; Cabezas-Cruz *et al*. 2019).

Examining the morphological, biochemical, clinical, and biological properties of the bacterial strains is possible with the conventional culturing approach. However, it was challenging to use this method for bacterial diversity studies because of their unique needs for growth in a lab setting. For the microbial diversity analysis, culturing individual bacteria is a strenuous task (Salmonová and Bunešová 2017; Yadav *et al*. 2024). The species discrimination power of biochemical tests of bacterial cultures is minimal. The *16S rRNA* sequencing often provides genus-level identification. We considered the colony morphology and biochemical reactions, and also the *16S rRNA* sequencing to confirm the bacterial genera. The *16S rRNA* gene sequences with a base pair homology greater than 99% with the representative strains on the NCBI nucleotide database were identified up to the genus level. Even though NGS methods could solve this problem, the species-level identification of bacterial strains solely depends on the reference sequences available in the NCBI database. The NGS data cannot provide the essential details of a bacterial strain, such as the morphological features, pathogenicity, antibiotic resistance, and clinical utility. Revolutionizing surveys of microfauna with

the help of metagenomic methods can provide new insights into the exploration of bacterial fauna associated with each tick species as well as environmental samples (Greay *et al*. 2018; Muhamad *et al.* 2020). More elaborated studies are required to explore the bacterial strains and their species-level identification. So, these findings can aid as a preliminary data set in monitoring tick-borne diseases from a larger viewpoint, as envisioned from "one health perspective" (Cunningham *et al*. 2017).

CONCLUSION

The analysis of the bacterial fauna associated with the cattle tick *R. annulatus* using culturable and NGS technologies showed the presence of 19 bacterial phyla. Three phyla were obtained by the culturable method. Eight genera of bacteria identified during the present study were all opportunistic pathogens. There exists a large literature gap in exploration of tick-associated bacteria. This work highlights the importance of persistent surveillance of tick-associated microbiomes with high-throughput molecular techniques to reduce the risks of vector-borne diseases effectively. This is the first attempt to explore bacterial diversity utilizing culturable and NGS techniques in *R. annulatus* ticks from India.

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میکروبیوم باکتریایی تین‌های *Rhipicephalus* (*Boophilus*) *annulatus* (Acari: Ixodidae)

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چکیده

علیرغم نقش تین‌ها در بسیاری از بیماری‌های عفونی، بررسی‌ها در مورد تنوع باکتری‌ها در گونه‌های تین‌ها محدود است. هیچ مقاله علمی در مورد بررسی میکروبیوتای باکتریایی مرتبط با تین‌های *Rhipicephalus* (*Boophilus*) *annulatus* (Say) در هند در دسترس نیست. در این بررسی، تلاش شد تا داده‌های دقیقی در مورد زیاگان باکتریایی مرتبط با *R. annulatus* از طریق روش‌های کشت سنتی و مستقل از محیط پرورش (توالی‌یابی نسل بعدی – NGS) جمع‌آوری شود. دو مکان با اهمیت مشترک بین انسان و دام در منطقه گهات غربی انتخاب شدند. نمونه‌های تین از گاوهای چرنده (*Bos indicus*) از ژوئن ۲۰۲۳ تا سپتامبر ۲۰۲۳ جمع‌آوری شد. روش کشت سنتی، سه شاخه و روش NGS ۱۹ شاخه را نشان داد. روش کشت سنتی، اکتینوباکتری‌ها را به عنوان فراوان‌ترین شاخه (۵۷/۱۴٪) و پس از آن پروتئوباکتری‌ها (۲۸/۵۷٪) و فیرمیکوت‌ها (۱۴/۲۸٪) گزارش کرد. بر اساس داده‌های NGS، شاخه فیرمیکوت‌ها (۳۵/۸۱٪) فراوان‌ترین شاخه با تفاوت‌هایی در فواصل اطمینان ۹۵٪ بود. این بررسی در مجموع هشت جنس باکتریایی با پتانسیل بیماری‌زایی را به دست آورد. با توجه به اهمیت پزشکی سویه‌های باکتریایی شناسایی شده در عفونت‌های مرتبط با مراقبت‌های بهداشتی (HAI)، این مطالعه بر نیاز به پژوهش‌های بیشتر در مورد میکروبیوم‌های مرتبط با کنه تأکید می‌کند.

واژگان کلیدی: روش کشت سنتی، هند، کرالا، توالی‌یابی نسل بعدی، تین.

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