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The midgut bacterial flora of the hard tick *Hyalomma anatolicum* (Acari: Ixodidae) from South India as determined by molecular analyses

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Abstract: The midgut bacterial flora of post-blood meal and starved or digested blood meal of hard ticks (*Hyalomma anatolicum*) from goats were analyzed using BOX PCR, ERIC PCR and *16S rDNA* gene sequencing. BOX PCR and ERIC PCR showed dissimilar band patterns between microbial isolates from the midgut of the ticks. In total, 9 bacterial species belonging to 2 genera (*Bacillus* and *Staphylococcus*) were identified in the midgut of both types (post-blood meal and digested blood meal) of tick by using *16S rDNA* gene sequencing. The majority of the midgut bacterial species were *Staphylococcus* sp. (90%) and there was a high frequency of 3 species of *Staphylococcus* (*S. chromogenes*, *S. epidermidis*, and *S. gallinarum*). Phylogenetic analysis based on *16S rDNA* gene sequences showed that all midgut isolates were monophyletic and showed high-frequency relationships with *S. chromogenes* and *S. epidermidis*. Further study is needed to understand the relationship between midgut bacterial community and tick–pathogen interaction.

Key words: Midgut bacterial flora, *Hyalomma anatolicum*, *16S rDNA*, molecular analysis

1. Introduction

Ticks are regarded as worldwide vectors of disease causing pathogens in animals. The hard tick (*Hyalomma anatolicum*) hinders cattle production in tropical regions and the midgut microbial flora of this tick in cattle remains largely unexplored. The hard tick is the most prevalent in India and cattle serves as the main host of this species (1). This tick develops behavioral or morphogenetic diapause in cold climates (2) but can multiply throughout the year in hot climates (3). *H. anatolicum* transmits various bacterial species of *Babesia*, *Anaplasma*, *Ehrlichia*, *Theileria*, and *Trypanosoma*, as well as several viruses (4,5). In India around 10 million cattle are at risk of tropical theileriosis transmitted by *H. anatolicum* with an annual economic loss of US $800 million (6). Its blood feeding behavior depends on the type of host. Thus, it undergoes a typical 3-host feeding cycle (larvae, nymph, and adult) on large domestic animals (3). As a result, pathogens are widely spread among domestic animals, making ticks important sources of zoonotic disease. Bacteria found in the guts of the tick may have an important role in the epidemiology of human or animal infectious diseases. Such bacteria may interfere with the development of medically important pathogens. For example, the symbiont *Sodalis glossinidius* may enhance the establishment of African trypanosomes in the tsetse fly midgut (7).

Recent works have examined the roles of symbiotic microorganisms in specific biological traits of several insect hosts (8) that should also be exploited in ticks and the feasibility of symbiotic control in countering vector-borne diseases (9). Recent studies clearly indicate that bacterial symbionts may influence specific traits of insect-host biology such as mating behavior in fruit fly (10), insecticide resistance in the bean bug, and fitness, nutrition (11), defense, and reproduction in a significant number of insect species (12). All these particular associations between symbionts and insects open interesting perspectives for tick-borne disease control; the relationships between bacteria and ticks merits study. In hard ticks, the most commonly isolated bacterial genera are *Stenotrophomonas*, *Staphylococcus*, *Pseudomonas*, and *Bacillus* and their relationships with the host are ambiguous (13). Although the role of *H. anatolicum* in the transmission of a wide range of human or animal pathogens has been intensively studied in terms of control strategies, less attention has been given to midgut bacterial flora of the hard tick. Hence, the aim of the present study was to analyze the composition of the midgut bacterial flora of the hard tick, *H. anatolicum*, by using molecular methods.

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2. Materials and methods

2.1. Tick sampling

The post-blood meal adult male and female ticks were collected using sterile forceps from goats in the rural area of Madurai, Tamil Nadu Province, India. The collected live ticks were kept in an aerated plastic container. In the laboratory, the collected ticks were identified. The 10 (5 males and 5 females) live post-blood meal ticks were directly taken for midgut dissection. The other 10 (5 males and 5 females) live post-blood meal ticks were kept for rearing in the laboratory for 15 days to digest the blood meal. Tick rearing was performed in the laboratory in a dark incubator at 29 °C and 85% relative humidity without feeding. Prior to dissection, each adult tick was sterilized externally with povidone-iodine in alcohol for 5 min followed by rinsing in sterilized double-distilled water. All midgut dissections were performed under sterile conditions.

2.2. Microbial culture

Midguts of 5 male and 5 female ticks post-blood meal and with digested blood meal were pooled separately and they were homogenized in 1 mL of sterile phosphate-buffered solution using autoclaved plastic tissue grinders. Homogenates were serially diluted and a 100-µL aliquot of sample was plated onto blood agar. The plates were incubated for 7 days at 28 °C. The isolated bacteria were streaked onto the appropriate medium at least 3 times before identification to ensure that pure cultures were obtained. The analyzed strains were maintained in a 1.5-mL Eppendorf tube containing 500 µL of pure cultures and 500 µL of 30% glycerin with 70% LB broth at –80 °C. The isolated strains were then subcultured onto nutrient broth (NB) for further analyses. The isolated bacterial colonies were picked on the basis of morphological features like size, shape, elevation, opacity, surface, texture, and margin yielding bacterial isolates from *H. anatolicum*. Bacterial genera and probable species were determined by comparing the results with Bergey's manual (14).

2.3. Template preparation for microbial fingerprinting analysis

The bacterial colonies picked from the subcultured plates and inoculated in 1 mL of NB were kept shaking for 3 h at 200 rpm and 37 °C. After turbidity development in the tubes, the tubes were centrifuged at 10,000 rpm for 10 min. The pellet obtained was subjected to treatment with 50 µL of colony lysis solution (1% Triton X-100, 20 mM Tris HCl of pH 8.0, 2 mM EDTA of pH 8.0). The mix was vortexed for a few seconds and heated at 65 °C for 15 min, and then 5 µL from each tube was transferred as the template for microbial fingerprinting analysis.

2.4. BOX PCR amplification

BOX PCR was performed to identify the microbial foot prints of BOX elements in the specific isolate. The reaction mixture contained 5 µL of 10X PCR buffer, 10 mM dNTPs mixture (2.5 mM each), 1.0 µM upstream primer (5'-ctacggcaaggcgacgctgacg-3'), 1.5 U of Taq DNA polymerase, and 50 ng of template. The amplification was carried out with 50 µL of the final volume in an Applied Biosystems thermal cycler (Germany). The DNA was subjected to initial denaturation at 94 °C for 4 min and final denaturation at 94 °C for 1 min, and it was annealed for 2 min at 60 °C and the reaction was extended to 2 min at 74 °C. The steps were programmed to repeat for 36 cycles with the final extension step at 74 °C for 5 min. Amplification products were visualized on 1% agarose gels stained with ethidium bromide.

2.5 ERIC PCR amplification

Enterobacterial repetitive intergenic consensus (ERIC) sequence primer amplification was used to determine the significant diversity and compare the fingerprints in the gut community. A 25-µL reaction mixture contained 1.0 µM ERIC upstream primer (5'-atgtaagctcctggggattcac-3') and downstream primer (5'-aagtaagtgactggggtgagcg-3'), 10X PCR buffer, 2.0 µL of 10 mM dNTPs mixture (2.5 mM each), 1.5 U of thermostable Taq DNA polymerase, and 50 ng of template DNA. The reaction was carried out with the following conditions: initial denaturation at 94 °C for 4 min, final denaturation at 94 °C for 1 min; it was annealed for 2 min at 60 °C and the reaction was extended at 74 °C for 5 min. Amplified products were visualized on 1% agarose gels stained with ethidium bromide.

2.6. DNA extraction

The genomic DNA was extracted from the isolated bacterial colonies using a protocol described by Moore et al. (15). The 24-h-old cultures of bacterial isolates were taken in microcentrifuge tubes. The tubes were centrifuged at 10,000 rpm for 10 min. The pellet was mixed completely with 90 µL of 10% SDS. The tubes were incubated at 37 °C for 1.5 h. To each tube was added 150 µL of 5 M NaCl prior to the addition of 100 µL of 10% cetyltrimethylammonium bromide in 5 M NaCl. The sample was mixed thoroughly and incubated at 65 °C for 30 min. DNA was extracted with an equal volume of chloroform, isoamyl alcohol, and phenol (25:24:1). DNA was precipitated with 70% ethanol and recovered by centrifugation. Pellets were suspended in 20 µL of TE buffer. Finally, the DNA bands were visualized in a gel documentation unit; the DNA concentration was determined by measuring the absorbance ratio at 260/280 nm, and the DNA suspension was stored at –20 °C until it was used for PCR and further analysis.

2.7. *16S rDNA* Amplification

To amplify the *16S rDNA*, the universal eubacterial primers 27F and 1492R were used. The reaction was carried out with the following condition using the forward primer 27F (5'-agagtttgatcctggctcag-3') and the reverse primer 1492R (5'-tacggctaccttgttacgactt-3'). The final volume of the mix was 25 µL, containing 5 µL of 10X reaction buffer, 2.5 µL of 10 mM dNTP mixture, 1 µL of 10 µM forward and reverse primer, 2.5 U of Taq polymerase, and 50 ng of template DNA. With initial denaturation at 94 °C for 3 min and cycled for 36 reactions with denaturing the template for 30 s at 94 °C and annealing at 55 °C for 1.5 min, the reaction was extended for 2.5 min at 72 °C and then extended finally for 10 min at 72 °C. To control for the presence of contaminating nucleic acids, water samples without template DNA were run in parallel. Amplification products were visualized on 1% agarose gels stained with ethidium bromide and then purified using the HiYield Gel/PCR-DNA Extraction Kit (Real Biotech Corporation, Taiwan). Finally, purified PCR products were sequenced with an automated DNA sequencer, Model 3500 (Applied Biosystems, USA).

All sequences were compared with *16S rDNA* gene sequences in the GenBank database using BLASTn search. Isolates were identified when their *16S rDNA* sequences shared ≥95% homology with complete *16S rDNA* sequences found in GenBank. For phylogenetic analysis, contigs were assembled and edited in MUSCLE v3.7 (16), and consensus sequences was exported in FASTA format for alignment. An improved version of the neighbor-joining (NJ) algorithm of BIONJ was implemented (17) using a Kimura 2-parameter model with gamma distributed (G). It is an improved nodal support for the NJ tree calculated with 1000 bootstrap replications with partial deletion using PHYLIP v3.2 (18).

3. Results

The bacterial culture in BA medium indicates that 29 and 33 bacterial colonies were formed in the post-blood meal midgut of adult males and females, respectively, while 21 and 24 bacterial colonies were formed in adult male and female isolated from the midguts of ticks that had digested the blood meal or starved. From male and female ticks post-blood meal or those that had digested blood meal, 21 similar and 9 dissimilar colonies were grown in the plates selected randomly. Of these, the 9 dissimilar bacterial colonies were separated based on their morphological and biochemical characterization. Furthermore, to characterize the selected colonies, they were subjected to molecular analyses.

The 9 dissimilar colonies formed in the culture plates were isolated. To test the variability of the dissimilar colonies, we used ERIC PCR and BOX PCR methods. The results of BOX PCR showed that the banding patterns ranged from 250 to 3000 bp and amplified products of the 9 dissimilar colonies loaded in the gel formed identical and different band patterns (Figure 1). This led us to perform ERIC amplification; the banding pattern of ERIC amplification also ranged from 250 to 3000 bp. In contrast to BOX PCR, ERIC PCR showed dissimilar band patterns for each isolate and the disparity led us to conclude the presence of 9 different colonies in the midgut of *H. anatolicum* (Figure 2).

The results of *16S rDNA* analysis of 9 different colonies in midgut isolates of *H. anatolicum* are presented in the Table. A total of 9 bacterial isolates were sequenced and their closest matches are shown, as determined with FASTA searches. The 9 identified species belong to 2 genera (*Staphylococcus* and *Bacillus*), of which the majority of species belong to the genus *Staphylococcus* (90%). Strains identified as belonging

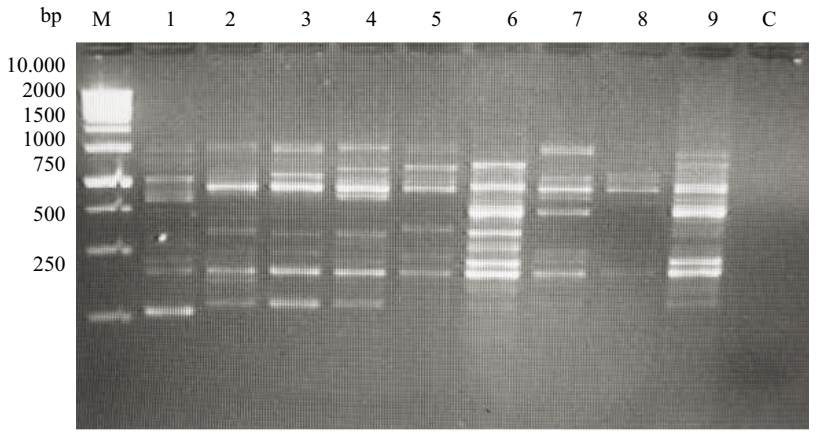

Figure 1. BOX PCR amplification of the culturable isolates of midgut in *H. anatolicum* (M- marker, C- control).

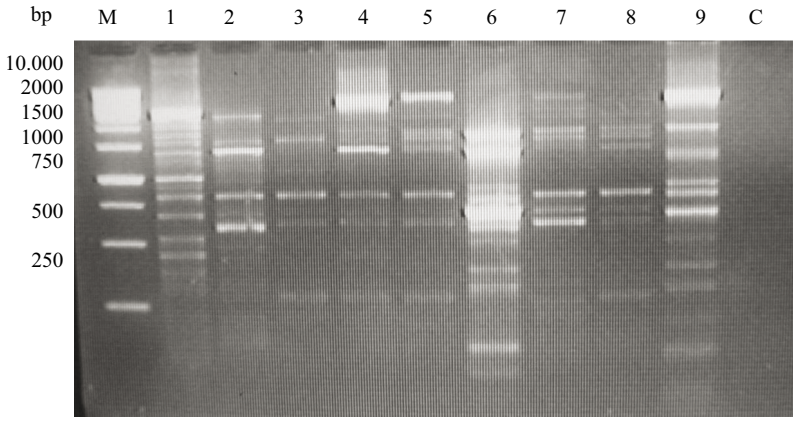

Figure 2. ERIC PCR amplification of the culturable midgut isolates in *H. anatolicum* (M- marker, C- control).

Table. List of bacteria isolated from the midgut of *H.* (*anatolicum*) *anatolicum*.

GenBank accession no.	Length	Closest match in NCBI-GenBank	Similarity
JN411550	931 bp	*Bacillus subtilis*, *Bacillus methylotrophicus*, *Bacillus amyloliquefaciens*	96%
JN411551	959 bp	*Staphylococcus* sp.	97%
JN411552	923 bp	*Staphylococcus epidermidis*	94%
JN411553	934 bp	*Staphylococcus* sp., *Staphylococcus hominis*, uncultured bacterium	96%
JN411554	940 bp	*Staphylococcus epidermidis*, uncultured bacterium	96%
JN411555	931 bp	*Staphylococcus chromogenes*	96%
JN411556	760 bp	*Staphylococcus gallinarum*, *Staphylococcus* sp., uncultured bacterium	92%
JN411557	946 bp	*Staphylococcus* sp., Actinomycetales bacterium, uncultured bacterium	96%
JN411558	935 bp	*Staphylococcus epidermidis*, *Staphylococcus* sp.	96%

to *Staphylococcus* sp. proved to be the most common gram-positive bacteria of *H. anatolicum*. However, most of the strains were isolated with a high frequency and they existed in only one of this tick species, e.g., *S. chromogenes*, *S. epidermidis*, and *S. gallinarum*.

The phylogenetic relationships of midgut bacterial isolates were evaluated using the *16S rDNA* gene sequence. Sequence alignments consisted of 729 positions. Of these characters, *16S rDNA* made up to 959. The 3 recovered topologies were almost identical, differing in the relative position of midgut isolates of *H. anatolicum*. The consistency index and retention index were 0.4277 and 0.6891, respectively. The consensus tree of BIONJ contained three polytomies (Figure 3). Phylogenetic analysis revealed that all midgut isolates (9 species) were monophyletic (bootstrap value: 92%–100%). BIONJ analysis recovered highly supported relationships among 8 species isolated from the midgut of *H. anatolicum* as formed in the first clade. Another midgut isolate of *Bacillus* sp. formed a third clade and the sister group was *Bacillus subtilis*.

4. Discussion

The results of microbial culture revealed that post-blood meal tick midgut had a higher number of bacterial colonies than the digested blood meal tick midgut. This finding agreed with the report of Li et al. (19) that, after a blood meal, the bacterial populations in the tick's midgut often undergo rapid massive expansions. The bacterial populations of the post-blood meal midgut of the vector are increased as much as 10,000-fold in triatomines and 70- to 16,000-fold in mosquitoes (20). In both post-blood meal and digested blood meal midguts of the ticks, the female's midgut had a higher number of bacterial colonies than male's midgut. This may be due to either a large

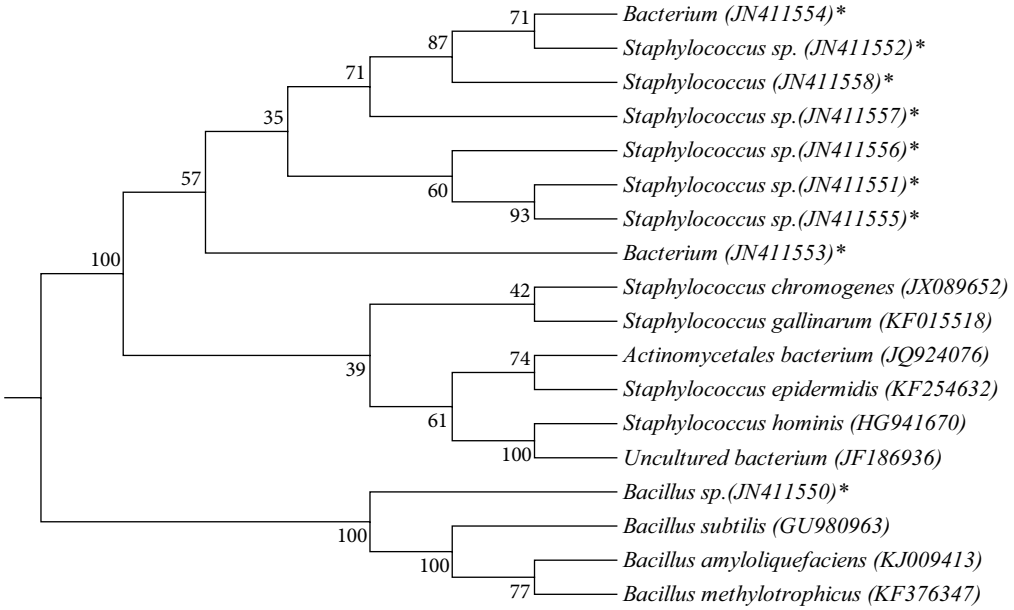

Figure 3. Neighbor-joining phylogenetic tree of *16S rDNA* gene sequences showing the relationship between midgut isolates of *H. anatolicum* and 96% similarity of outgroup species. Bootstrap values shown above nodes. *: Sequenced in this study.

quantity of blood or feeding time. Female ticks usually feed for 8 to 12 days until full and increase their weight by 100 times (21).

Nine species belonging to 2 genera were characterized on the basis of their morphological and biochemical characteristics, which were combined with the BOX PCR, ERIC PCR, and *16S rDNA* sequence analyses. Molecular methods provide an efficient opportunity to analyze bacterial communities in ticks, avoiding the need for intensive culture-based techniques, and allow the identification of species (22). Nine bacteria were identified from the midgut of *H. anatolicum* to the generic level, including *Staphylococcus* and *Bacillus*. Similar results were observed in the closely related tick species *Rhipicephalus decoloratus*, *R. geigyi*, and *R. microplus* (22,23).

In the present study, most of the strains of *Staphylococcus* isolated from midguts showed high-frequency relationships with *S. chromogenes* and *S. epidermidis* in adult male and female ticks. Due to spatial variation, the current midgut isolates of *Staphylococcus* may have high genetic diversity and showed 96% similarity with *S. chromogenes* isolated from *R. microplus* collected in Australia (13). *S. chromogenes* is part of the natural skin flora of cattle, but it can cause mastitis, and in pigs it may provoke exudative epidermitis (24,25). *S. epidermidis* is found ubiquitously on healthy human skin and mucosal surfaces and its virulence factor causes neonatal infection (26). *S. gallinarum* is commonly found in poultry and is unusual in humans; it causes septicemia in humans (27). Another midgut strain of *Bacillus* sp. is related to *B. subtilis*. The common bacterium of *B. subtilis* is found in soil, water, and plants, and it is an important source of industrial enzymes (28). *B. subtilis* was also isolated from the intestine of poultry in Malaysia (29).

Elucidating the taxonomic composition of gut bacteria facilitates the understanding of phylogenetic relationships between symbionts and the evolutionary biology of their association with tick hosts (30). Phylogenetic analysis showed that the midgut bacterial flora of *H. anatolicum* formed a separate clade and was poorly related with other strains taken as outgroup taxa based on *16S rDNA* gene sequences. This may be due to insufficient information available on the midgut flora of tick from the host of goats. To our knowledge, this is the first report of the bacterial flora in tick midguts from the host of goats, although there are a few reports on ticks harboring a wealth of microorganisms. The overall results suggest that *Staphylococcus* spp. are the common bacteria in the midgut of *H. anatolicum* and they might be related to the development of medically important pathogens in ticks. Further study is required to understand the relationship between midgut flora and tick–pathogen interactions.

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