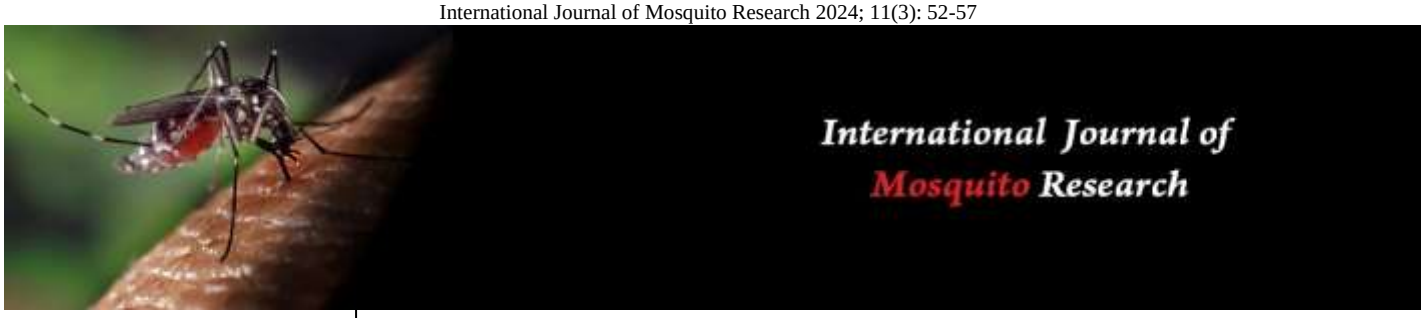

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Isolation of *Staphylococcus gallinarum* from the ovary of *Armigeres subalbatus*

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Abstract
Mosquitoes, as vectors of various pathogens, pose significant threats to human health worldwide. Among them, *Armigeres subalbatus* stands out for its role in transmitting diseases such as Japanese encephalitis and filariasis. Despite its importance, little is known about the microbial communities within its ovaries. In this study, bacteria from the ovaries of *Armigeres subalbatus* were isolated, characterised and identified using conventional and molecular techniques. The predominant species identified was *Staphylococcus gallinarum*. The findings of the study shed light on the ovarian microbiota of *Armigeres subalbatus* and its potential implications for mosquito biology and disease transmission dynamics. Understanding these interactions is crucial for developing innovative strategies for vector control and disease prevention.

Keywords: Mosquitoes, *Armigeres subalbatus*, *Staphylococcus gallinarum*, ovary, 16s rRNA sequencing

Introduction
Mosquitoes, belonging to the order Diptera, family Culicidae, are renowned vectors of numerous pathogens that pose significant threats to human health globally. Within this diverse family, several genera, including *Aedes*, *Anopheles*, and *Culex*, stand out for their ability to transmit a wide range of diseases [1]. Among the diseases transmitted by mosquitoes, malaria, dengue fever, chikungunya, Japanese encephalitis, Zika, and West Nile fever are some of the most notable, causing substantial morbidity and mortality worldwide [2].

Armigeres subalbatus is a species of mosquito found across Asia and the Pacific region. Even though *A. subalbatus* species is considered as harmless compared to other mosquito species it plays a significant role as a vector for various diseases, including Japanese encephalitis, filariasis, and certain arboviruses. Its vector status is found to be significant in the transmission of Japanese encephalitis, which lead to severe neurological complications and also in the transmission of filarial parasites, contributing to the spread of lymphatic filariasis in endemic regions [3-5]. Its ability to transmit multiple pathogens underscores the importance of understanding its ecology and implementing effective control measures to mitigate disease transmission and reduce the burden of mosquito-borne illnesses in affected populations.

The intricate relationship between mosquitoes and the pathogens they transmit is further complicated by the diverse microbial communities inhabiting various anatomical niches within the mosquito. These microbial communities, collectively known as the mosquito microbiota, encompass a multitude of bacteria, fungi, viruses, and other microorganisms that interact with the mosquito host and potentially influence disease transmission dynamics [6, 7]. While much attention has been focused on the midgut microbiota, the microbial inhabitants of other anatomical regions, such as the salivary glands, ovaries and malphigian tubules remain relatively unexplored.

The ovaries of female mosquitoes play a pivotal role in reproduction, serving as sites for egg development and maturation. Despite their importance, the microbial communities residing within the mosquito ovaries, particularly the ovarian bacteria, have received limited attention in scientific research [8].

Understanding the composition, diversity, and functional roles of ovarian bacteria is crucial for unravelling their potential impact on mosquito biology, reproductive fitness, and vector competence.

The main objective of the present study is to address this knowledge gap by isolating and characterising bacteria from the ovaries of female mosquitoes. By elucidating the ovarian microbiota composition and its potential implications for mosquito biology and disease transmission, these findings will contribute to a deeper understanding of the intricate interactions between mosquitoes, pathogens, and microbial symbionts, with implications for the development of novel strategies for vector control and disease prevention.

Materials and Methods

Collection of mosquitoes

Adult *A. subalbatus* mosquitoes were observed to be active predominantly during the evening and nighttime hours. They were frequently observed in large numbers during dusk, characterised by their higher-pitched chirping. Several adult female mosquitoes were randomly collected using insect nets from human settlements in Dharmadam, Thalassery (latitude 11°46'37" N, longitude 75°28'10" E) and the mosquitoes were transported to the laboratory in sterile containers. As the collection sites were not designated as conservation areas, and Aedes subalbatus is not classified as a threatened or endangered species, obtaining formal permission for the collection was considered unnecessary.

Identification of mosquito species

The collected mosquitoes were anaesthetized in the laboratory with chloroform and their morphological characteristics were noted under a stereo-zoom microscope for identification. Adult Armigeres mosquitoes bear resemblance to species from other Aedine genera, although they typically exhibit larger size [5]. These species are characterised by a proboscis that is slightly curved downward and flattened laterally along with eyes positioned ventrally and bearing two rows of scales [5, 9].

Dissection of Ovary

Before dissection, female mosquitoes were anaesthetized with chloroform. Subsequently, each female mosquito was positioned on a glass slide with a droplet of 1X phosphate-buffered saline (PBS) solution under a stereoscopic microscope (Radical, Model No. RSM-9). Using two needles, an incision was made between the sixth and seventh tarsal segments of the abdomen to remove the ovaries into the PBS solution [10].

Isolation of ovarian Bacteria

The homogenized ovary extract was diluted tenfold with PBS. Around 100 µl of the diluted homogenate was then spread onto nutrient agar plates. These agar plates were prepared by dissolving 5.6g of nutrient agar in 200 ml of water at 50 ℃ for 5 minutes in a water bath, followed by autoclaving at 15psi for 10 minutes. Afterwards, the agar plates were uniformly spread using a spreading rod and then incubated at 37 ℃ for 24-48 hours. All isolation procedures were conducted under sterile conditions, and as a negative control, blood agar plates poured with 100 µl of sterile PBS water were maintained. Bacterial colonies detected were subcultured [11].

Gram staining

Bacterial cultures were streaked onto glass slides using a sterile loop to create a thin bacterial smear. The bacterial smear was air-dried and then heat-fixed by passing the slide through a flame three times, ensuring gentle heating without overheating the slide. The fixed bacterial smear was flooded with crystal violet (CV) solution for one minute, allowing the stain to cover the entire smear. Excess crystal violet solution was gently rinsed off the slide using distilled water. Gram's iodine solution was applied to the bacterial smear for one minute, serving as a mordant to enhance the interaction between the crystal violet and bacterial cell wall. The slide was gently rinsed with ethanol or acetone until the runoff became colourless. Decolorization was carefully monitored to prevent over-decolorization. The bacterial smear was then flooded with safranin solution for 30-60 seconds, staining the decolourized bacteria with a contrasting colour. Excess safranin solution was rinsed off with distilled water, and the slide was gently blotted dry with absorbent paper. The prepared slide was examined under oil immersion microscopy at 40x and 100x magnification. Bacterial cells were observed for their colour reaction to determine Gram stain results [12, 13].

Genomic DNA Isolation and PCR Amplification of 16s rRNA Gene

The bacterial genomic DNA was extracted using the HiPurA bacterial DNA isolation kit from HIMEDIA, following the manufacturer's protocol. The bacterial cells were lysed using a lysis buffer containing lysozyme and proteinase K, and the DNA from the lysed cells was purified using silicate columns. The purified DNA was then eluted from the column using TE buffer (pH 8). This eluted DNA served as the template for PCR amplification of the 16SrRNA gene, using the fD1 and rP2 primers [14, 15].

PCR amplification was conducted using the Sure Cycler 8800 automated Thermal cycler from Agilent Technologies, USA. The PCR reaction mixture comprised 25 µl of 2x premix of Emarald AMP GT PCR master mix, 2 µl each of forward and reverse primers (5 µM each), 1 µl of template DNA, and sterile dH2O added up to a total volume of 50 µl. The PCR temperature profile included an initial denaturation at 95 °C for 5 minutes, followed by 30 cycles of denaturation at 95 °C for 1 minute, annealing at 59 °C for 1 minute, and elongation at 72 °C for 2 minutes. A final extension step at 72 °C for 5 minutes concluded the amplification process. The reaction mixture was stored at 4 °C after completion [11].

The amplification was confirmed by analyzing 5 µl of the PCR product on a 1% ethidium bromide-stained agarose gel using a gel documentation system (Bio-Rad EZ Gel Documentation System) [15]. The remaining 45 µl of the PCR product was retained for sequencing. Subsequently, the amplified PCR product was purified using the StrataPrep PCR purification kit from Agilent Technologies, USA, according to the manufacturer's instructions.

DNA Sequencing

The PCR product, once purified, underwent Sanger

sequencing [16] at Biokart in Bangalore, with sequencing carried out from both ends. Subsequently, the forward and reverse sequences were aligned and merged to produce a consensus sequence using Sequencher 5.3 software from Gene Codes Corporation in Ann Arbor, Michigan, USA. This consensus sequence was then compared for similarities using the BLASTn program on NCBI [17]. Similar sequences were selected from the 16S rRNA gene database on GenBank to identify the bacterium based on the closest matches. Additionally, the isolated sequence was cross-referenced using the Ez Taxon server (http://www.ezbiocloud.net/eztaxon). Homologous sequences retrieved from GenBank were utilized to construct a phylogenetic tree using the Maximum Likelihood method in MEGA XI software [18].

Results

By repeated bacterial culturing, translucent large colonies were observed frequently. The colony was subcultured, analyzed by PCR, and sequenced. A consensus DNA sequence of 1133bp was obtained from an ovarian bacterial isolate of *A. subalbatus.*

```
>___A02_B1_A01.ab1
GGTGCGGGTGCTATACATGCAAGTCGAGCGAACAGATAAGGAGCTTGCTCCTTTGACGTTAGCGGCGGACGGGTGAGTAA
CACGTGGGTAACCTACCTATAAGACTGGAATAACTCCGGGAAACCGGGGCTAATGCCGGATAACATATAGAACCGCATGG
TTCTATAGTGAAAGATGGTTTTGCTATCACTTATAGATGGACCCGCGCCGTATTAGCTAGTTGGTAAGGTAATGGCTTAC
CAAGGCGACGATACGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGG
CAGCAGTAGGGAATCTTCCGCAATGGGCGAAAGCCTGACGGAGCAACGCCGCGTGAGTGATGAAGGGTTTCGGCTCGTAA
AACTCTGTTATTAGGGAAGAACATATGTGTAAGTAACTGTGCACATCTTGACGGTACCTAATCAGAAAGCCACGGCTAAC
TACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGTAGGCGGTTT
CTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAAACTTGAGTGCAGAAGAGGAAAG
TGGAATTCCATGTGTAGCGGTGAAATGCGCAGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACT
GACGCTGATGTGCGAAAGCGTGGGGATCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGT
GTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAAC
TCAAAGGAATTGACGGGGACCCGCACAAGCGGTGTAGCATGTGGTTTTATTTCGAAGCAACGCGAAGAACCTTACCAAAT
CTTGACATCCTTTGACCACTCTAGAGATAGAGCTTTCCCCTTCGGGGGACAAAGTGACAGGTGGATGCATGATTGTCGTC
AGCCTCGTGTCGTGAGATGATGGGTTAAAGTCCCGCAACGAGCCGCAAACCCTTAGCTTAGTTGCCATCATTAAGTTTGG
GCCACCTCTCTAA
```

The alignment with the reverse complement of the reverse strand gives a consensus of 1133 bp length 16s rRNA gene database of GenBank, giving the following BLAST hit results.

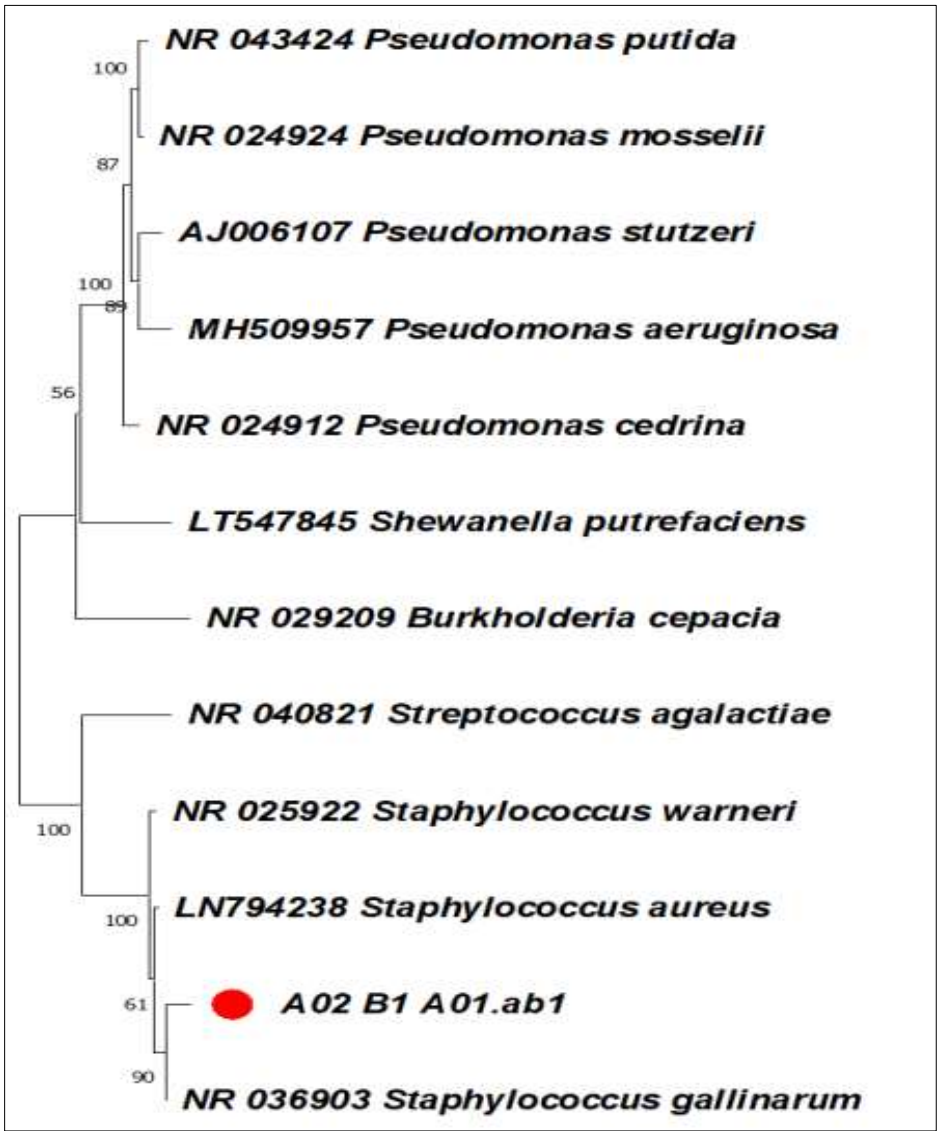
Serial No.	Accession No.	Hit taxon name	Percentage of similarity
1.	MN818730	*Staphylococcus gallinarum*	98.93
2.	MK977614	*Staphylococcus haemolyticus*	98.83
3.	MN851075	*Staphylococcus arlettae*	98.66
4.	MW453037	*Staphylococcus aureus*	98.66

BLAST hit table showing similarity of bacterial sequence with other 16s rRNA sequences of databases. The BLAST hit results showed 98.93 similarity to *Staphylococcus gallinarum.*

The sequence isolated was also compared in the Ez Taxon server (http://www.ezbiocloud.net/eztaxon) gives the following table.

Serial No.	Accession No.	Hit taxon name	Percentage of similarity
1.	D83366	*Staphylococcus gallinarum*	97.87
2.	D83361	*Staphylococcus cohnii*	97.24
3.	MT586030	*Staphylococcus borealis*	96.97
4.	AB009933	*Staphylococcus arlettae*	96.90
5.	AF004220	*Staphylococcus succinus*	96.81

From these tables, it was confirmed that the bacteria isolated from the ovary of *A. subalbatus* is *Staphylococcus gallinarum.* The homologous sequences retrieved from GenBank were used for the construction of a phylogenetic tree using the Maximum likelihood method in the MEGA XI application.



Evolutionary relationships of taxa

The evolutionary history was inferred using the Neighbor-Joining method [19]. The optimal tree is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) are shown next to the branches [20]. 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 Maximum Composite Likelihood method [21] and are in the units of the number of base substitutions per site. This analysis involved 12 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1560 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 [18].

Serial No.	Accession No.	Scientific name as in GenBank	Percentage similarity	Geographic Location
1.	NR043424	*Pseudomonas putida*	79.91	Japan
2.	NR024924	*Pseudomonas mosselli*	80.26	France
3.	AJ006107	*Pseudomonas stutzeri*	80.17	Germany
4.	MH509957	*Pseudomonas aeruginosa*	80.27	Iraq
5.	NR024912	*Pseudomonas cedrina*	81.31	USA
6.	LT547845	*Shewanella putrefaciens*	80.49	Denmark
7.	NR029209	*Burkholderia cepacia*	77.99	USA
8.	NR040821	*Streptococcus agalactiae*	85.49	Japan
9.	NR025922	*Staphylococcus warneri*	96.44	USA
10.	LN794238	*Staphylococcus aureus*	96.48	Saudi Arabia
11.	NR036903	*Staphylococcus gallinarum*	98.66	Japan

From this cladogram, it is concluded that the identified bacterial sequence is similar to *Staphylococcus gallinarum*. When it was gram-stained the result was a gram-positive bacteria, consisting of single, paired, and clustered cocci.

By analyzing the cladogram, the bacteria was found to be monophyletic with *Staphylococcus gallinarum* (NR036903).

It was paraphyletic with *Staphylococcus warneri* (NR025922), *Staphylococcus aureus* (LN794238) and *Streptococcus agalactiae* (NR040821), and polyphyletic with, *Burkholderia cepacia* (NR029209), *Shewanella putrefaciens* (LT547845), *Pseudomonas putida* (NR043424), *Pseudomonas mosselli* (NR024924), *Pseudomonas stutzeri* (AJ006107), *Pseudomonas aeruginosa* (MH509957) and *Pseudomonas cedrina* (NR024912).

Discussion

The investigation of ovarian microbiota in mosquitoes represents a relatively underexplored area within vector biology. Despite recent advancements in molecular techniques, comprehensive studies on the diversity and composition of bacterial communities associated with mosquito ovaries remain limited. Existing studies suggest that the bacterial abundance and diversity in the salivary glands and ovaries are comparatively lower when compared to the midgut [22]. The present investigation aimed to address this gap by employing both conventional culture-based methods and molecular sequencing approaches.

The previous reports highlight Actinobacteria and Proteobacteria as the predominant bacterial phyla inhabiting mosquito ovaries. The ovaries are particularly intriguing due to their role as sites for virus replication, as well as the potential influence of specific bacteria on the colonization and displacement of other symbionts. This is of significant interest because certain bacteria may possess biotechnological and biological capabilities that could be harnessed for vector control purposes [23, 24].

S. gallinarum was found to be the predominant species within the ovaries of *A. subalbatus* specimens collected from human settlements in Thalassery. *S. gallinarum*, a gram-positive bacterium, is known for its propensity to form single, paired, or clustered colonies. Noteworthy is its resemblance in cell wall composition to Staphylococcus epidermidis, suggesting potential implications for host-microbiota interactions.

The inclusion of *S. gallinarum* in the ovarian microbiota of *A. subalbatus* adds to the limited repertoire of bacterial species associated with mosquito reproductive tissues. This novel finding underscores the necessity for further investigations into the ecological dynamics of mosquito-associated microbiota and their implications for vector-borne disease transmission. The ovarian microbiota of *Aedes aegypti* exhibits potential for the development of paratransgenesis strategies, given its ability for both paternal and maternal transmission [25].

Furthermore, the present study contributes to the broader understanding of *A. subalbatus* as a vector of filariasis. By elucidating the microbial communities residing within its ovaries, we provide insights into potential mechanisms influencing vector competence and disease transmission dynamics. This knowledge has significant implications for the development of targeted vector control strategies aimed at interrupting filarial disease transmission cycles.

The identification of *S. gallinarum*, a bacterium previously reported in human saliva, implicated in opportunistic infections, raises intriguing questions regarding its role as a potential commensal or pathogen in mosquito biology. The documented case of bacteremia in a patient with chronic hepatitis B virus infection highlights the need for further investigation into the epidemiological significance of *S. gallinarum* and its potential impact on human health [26].

Moving forward, future studies should focus on elucidating the mechanisms governing the colonization and persistence of ovarian bacteria in mosquitoes. In addition to this, efforts to characterize the functional roles of these bacteria in modulating mosquito physiology and vector competence are warranted. Such endeavours are essential for advancing our understanding of mosquito-microbiota interactions and informing the development of innovative strategies for vector-borne disease control.

Conclusion

The investigation into the ovarian microbiota of *A. subalbatus* sheds light on a previously overlooked aspect of mosquito biology. By identifying *Staphylococcus gallinarum* as a prevalent member of this microbial community, the present study highlights the intricate interplay between mosquitoes, their microbiota, and disease transmission dynamics. This study underscores the importance of considering microbial symbionts in vector biology research and emphasizes their potential as targets for novel vector control strategies. Moving forward, continued exploration of mosquito-associated microbiota promises to yield valuable insights into vector-borne disease ecology and pave the way for innovative approaches to mitigate their impact on human health.

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