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Molecular characterization and distance tree identification of unique environmental carryover proteobacteria *Mesorhizobium* sp. in the midgut of *Anopheles stephensi*

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Abstract
Recently, environmental carry over symbiotic bacteria has a significant attention in the role of microbes in mosquito gut. The gut microbes influence the transmission and development of pathogens. However, the effects of micro biota in guts made the parasites to proliferate with the host interaction. So identification of gut microbe is very important for vector control mainly on malarial mosquitoes. *Anopheles stephensi* is a primary vector for spreading malarial disease in Asia and most predominant in the Kancheepuram district, Tamilnadu, India. In this present investigation we found that unique environmental carryover soil Proteobacteria in midgut of *Anopheles stephensi* where malaria fever outbreak subtropical zone. Larvae of *Anopheles stephensi* mosquitoes were collected from kancheepuram district, Tamilnadu, India. The genomic DNA isolated and 16s rRNA gene sequencing performed for bacterial identification in midgut region of *Anopheles stephensi* mosquitoes and distance tree constructed to determine the origin. Our finding suggested that single existence of soil environmental *Mesorhizobium* Sp. in the gut. Most of the other bacterial genera in the study have been already identified. Scientists were under pressure to investigate further to know mechanism of the carryover environmental microbes to mosquitoes. This preliminary interesting report gives a path for the scientist to explore further to control the malarial vector disease.

Keywords: Malaria, vector, anopheles, 16s rrna, proteobacteria, distance tree

1. Introduction
Vector borne disease cause health issues in Asia and one of them is malaria which almost produce high mortality rate in recent years. The malaria spread by female *Anopheles stephensi* mosquitoes in the tropical area, risks influenced through rainfall, temperature and unplanned urbanization [1]. Frequently, the outbreak and seriously affected for malaria is only in South East Asia region. There is lot of control measures emerging to reduce the mortality level of malaria especially insecticide but other controlling approaches under process is para-transgenes is which defined at the symbiotic relationship producing bacteria [2]. Several researches evidently proved that a bacterium in the midgut of disease vector affects the host and interactions between the host and the pathogen influences the disease transmission[3]. The mosquito survival modulates the entire composition of midgut bacteria flora such as digestive enzyme, pH and food ingested by the host. Recently several laboratories focused on the identification of mid gut micro flora to control the disease. Therefore, we undertook this molecular study and focused only the single bacterium in the midgut the other bacteria were not focused because existed bacteria previously reported. The results of the present study may be useful to researchers to identify the mechanism of existence related to the vector control management.

2. Materials and Methods
2.1 Rearing of mosquitoes and midgut bacteria isolation
Fourth stage larvae of *Anopheles stephensi* mosquitoes were collected from 5 different places of kancheepuram district, Tamilnadu, India during the outbreak of the disease November-

December 2018. The collected mosquito's larvae were transported to lab in the sterilized condition and placed in the sterilized net cage for the development process. The mosquito sample was surface sterilized with ethanol for 5-10 minutes followed through PBS prior to separation process. The dissected midguts were sterilized using PBS. The sterilized gut homogenates was serially diluted and isolated the bacteria through pour plate method [4].

2.2 Morphology and biochemical identification

The bacterial colonies were identified based on morphological such as shape, staining, spore formation, motility [5] and biochemical characteristics such as indole, citrate, methyl red, oxidase and voges proskaur test[6]. And unique morphologically character colony was subculture for further molecular identification.

2.3 Isolation of DNA and 16s rRNA gene amplification

Overnight incubated bacterial culture was used for isolation of genomic DNA, the universal Himedia DNA kit was used and method adopted based on manufacturer instructions. The purified DNA was taken for PCR amplification using the 16S rRNA universal gene segment primer. The primer set 16SF (AGAGTTTGATCHYGGYTYAG) and 16 SR (ACGGCTACCTTGTTACGACTT) in 50 µl preparation mix. The amplification program set for the reaction, starts from initial denaturation at 95 °C for 3 min, then 33 cycles of 94 °C for 90 s, 50 °C for 35 s, 72 °C for 105 s continued by a finial extension 72 °C for 3 min. The amplified PCR product was quantified in 1.5% agarose gel electrophoresis with ethidium brodium dye (0.5µg/ml). The amplified PCR product further purified using HIMEDIA gel purification kit, quantified in 1% low melting agarose gel products and it was analyzed in ABI 310 genetic analyzer.

2.4 Sequence analysis and Distance tree construction

The nucleotide sequence was edited using BIOEDIT software and FASTA sequence was submitted to NCBI Genbank under accession number (MN544888) and NCBI database BLAST (http://www.ncbi.nlm.nih.gov/BLAST). For distance tree for the sequences was viewed using NCBI TREEVIEWER (https://www.ncbi.nlm.nih.gov/tools/treeviewer/) and construct neighbor joining phylogenetic cladogram tree with the identification based on sequence similarities [7].

3. Results and Discussion

The present study was carried out to detect an unique bacterium in midgut *Anopheles stephensi*. The bacterial colonies obtained from *Anopheles stephensi* mosquitoes midgut was morphologically identified and single colony was unique from other colonies. The mosquitoes midgut bacteria identification plays an important role in the vector parasite interaction [8]. The morphological identified colonies were gram negative bacteria may be phylum of Proteobacteria [9] and gram positive bacteria may be Firmicutes[10] and substrate mycelium with spore with filamentous in nature may be Actinobacteria[11] which was already reported in the midgut of the mosquitoes so data was not shown. The unique colony was morphologically identified as rod shaped, gram negative and non-spore forming bacteria. The biochemical characteristics of the unique colony showed indole, methyl red and vogesproskaur activity (Table 1). Our findings correlated with earlier report of *Mesorhizobium* Sp. Characteristics [12].

Table 1: Morphological and Biochemical characteristics for *Mesorhizobium* Sp

Characteristics	Activity
Morphology	
Gram Staining	-ve
Shape	rod
Spore	Non spore forming
Motility	Flagellated
Biochemical	
Indole	+
Methyl Red	+
Citrate	-
Vosges Pros Kaur	+
Oxidase	-

The single colony was further sub cultured for gene identification process. The isolated genomic DNA approximately 7 Mb size to the 1kb plus DNA ladder. The universal 16S rRNA gene primer was used and size of the unique environmental bacteria was approximately 1500bp to the relative 1kb marker (Figure 1). The sugarcane rhizosphere soil bacteria of *Mesorhizobium* 16S rRNA gene was identified and reported as 1500bp using universal primer [13]. The symbiosis bacteria play a major significant role for the association host interaction and disease progression. However, mosquito species have distinct microbiota in different organs which reflect the functions. The sequence was BLAST in the NCBI for the analysis. The query sequence was assessed and compared against the database related to the query coverage, percentage, e-value etc. Most often the coverage for the query denotes the percentage of the sequence length involved in the query region of alignment. The percentage identity denotes the percentage residue at the same position of aligned sequence. Whereas, e-value signify the alignments chance and space size in the database [14]. The sequence searches was restricted to the 16s ribosomal RNA sequences (Bacteria and Archaea) and high similar sequence (mega blast).

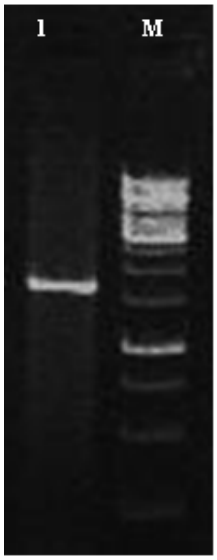

Fig 1: 16S rRNA sequencing gel image with 1kb marker;

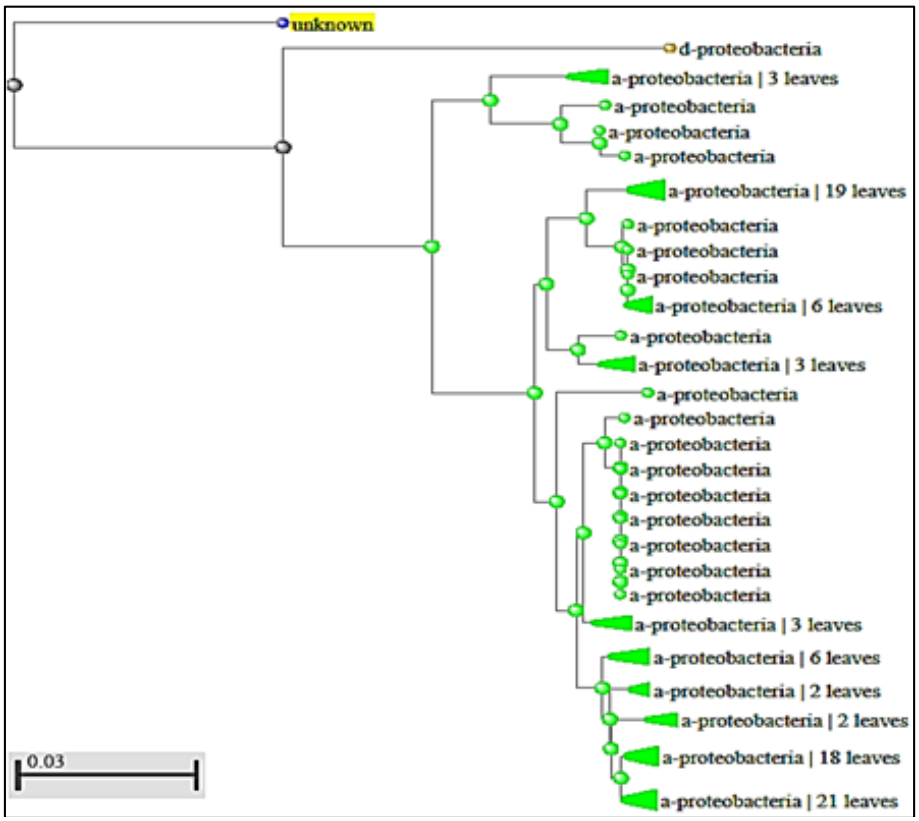

Fig 2: Neighbor joining Distance cladogram tree relationship for unknown which indicated MN544888 (*Mesorhizobium* Sp.).

The search reveals query coverage of 98% with 3e-119 and percentage identity of 82.40 as *mesorhizobium* strain *acaciae*. The strain was so unique which showed the identity less than 85% percentage similarity with other strains. Analysis revealed that the strains may be monomorphic in nature. *Mesorhizobium acaciae* bacteria most predominant in the leguminous plant soil but still unknown about the carryover mechanism from the environment to midgut region. The distance of *Mesorhizobium acaciae* strain KSE identified in NCBI BLAST TREEVIEWER using neighbor joining cladogram tree with maximum sequence difference of 0.75 clearly showed the ancestors evolution genera as proteobacteria (alpha and delta) with distance of 0.03 (Figure 2). Our findings correlated with earlier reports that the candidates of *Mesorhizobium*, *Phyllobacterium*, *Rhizobium* bacteria identified in the different organs of the mosquitoes [15]. *Mesorhizobium* Sp identified from the somatic and reproductive tissues of mosquito *Ae. Albopictus* and capable to transmissible pathogen replication [16]. Malaria transmitting mosquitoes are frequently linked to microbes mainly in the midgut. This may be modulate the mosquitoes vectorial capacity either inhibition or expression through unknown mechanism. The *Mesorhizobium* bacteria recorded in the *Culex* Sp and *Aedes* Sp. which gives an additional support for our findings [17]. Limited number of researches recorded that environmental bacteria carry over in mosquitoes but none of the research focused to know the carry over mechanism to mosquitoes and their role of bacterial infection to the human beings. The detection of this *Mesorhizobium acaciae* may be associated with other midgut bacteria possibly for better adaptability to the ecosystem. This report may give a debate to scientist to dig out the mechanism and role to control vector disease. Further analysis should be needed for the better understanding the invasion to mosquitoes and importance of paratransgenetic assessment to control mosquito borne diseases.

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