International Journal of Pharmacy and Biological Sciences
ISSN: 2321-3272 (Print), ISSN: 2230-7605 (Online)
IJPBS™ | Volume 8 | Issue 4 | OCT-DEC | 2018 | 360-368
Research Article | Biological Sciences | Open Access | MCI Approved| ज्ञान-विज्ञान विमुक्तये
|UGC Approved Journal |

THE STUDIES ON UNDERSTANDING OF MIDGUT BACTERIAL COMMUNITY OF *AEDES AEGYPTI* COLLECTED FROM TAMILNADU, INDIA- A METAGENOMIC APPROACH

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ABSTRACT

Metagenomics as a new field of research has been developed over the past decade to elucidate the genomes of the non-cultured microbes with the goal to better understand global microbial ecology on the one side, and on the other side it has been driven by the increasing biotechnological demands for novel enzymes and biomolecules. Ingestion of blood meal by female mosquitos triggers a series of physiological processes in the midgut and also exposes them to infection by these pathogens. The bacteria normally harbored in the mosquito's midgut are known to influence physiology and can also alter the response to various pathogens. Here, we studied the bacterial diversity found in midgut part of Aedes aegypti to understand the host and microbe interaction. The adult Aedes aegypti mosquitos were collected from Chidambaram and around area using ovitrops and midgut part was extracted moreover the DNA templates were isolated and amplified by PCR. The DNA amplicons were sequenced by Illumina MiSeq gene sequence. The total 179,310 reads were classified in to 209, the bacterial genera of Aeromonas, Klebsella, Acinetobacter, Staphylococcus, Bacillus and Pseudomonas predominantly found to be high when compared to the other bacterial genera. The present data strongly encourage further investigations to verify the potential role of the detected bacteria in mosquito for the transmission several vectoral diseases.

KEY WORDS

midgut, Aedes aegypti, Illumina, Aeromona and vectoral diseases.

INTRODUCTION

Mosquito borne diseases are dramatically affect public health and represent a major burden in terms of economy and development worldwide (WHO, 2011) Among the disease transmitting insects, the mosquitoes are the primary hosts for transmission of diseases like malaria, dengue, chikungunya, lymphatic filariasis, yellow fever etc., which together are responsible for several million deaths and hundreds of millions of cases every year (Chandel *et al*., 2013). There are approximately 500 *Aedes* species in the world (Arbain, 1990; Lee, 1999). The observations over 100 years have shown that the epidemiology of dengue varies a great deal with respect to both geography and time. Mosquito-borne diseases menace the living and livelihood of millions of people worldwide (Tamizhazhagan *et al*., 2017). This is due to not only to modifications in human ecology (population increase, urbanization, more frequent travel) but also the ecological adaptations of certain mosquito species

(Teng & Singh, 2001). One of the most difficult problems encountered with species that do not adapt readily to laboratory culture is getting the adults to mate and lay fertile eggs in captivity. No difficulty of this type has been encountered with *Aedes aegypti* (Gahan & Smith, 1964). The burden of dengue fever has increased drastically in the last few decades, and about 40% population living in more than 100 countries is affected. Dengue virus (DENV) is an RNA arbovirus and the causative agent of dengue fever and the more severe dengue haemorrhagic fever. There are four serotypes of DENV (DENV1-4), which together are estimated to infect 390 million people per year. The mosquito *aedes aegypti* are considered major public health problems. Mosquitoes spread many disease-causing viruses and parasites between people and other animals, including viral infections such as dengue and Chikungunya (Reiter *et al*., 2006; Delatte *et al.*, 2008). The mosquitoes *Aedes aegypti* ,*Stegomyia aegypti* (Reinert *et al*., 2009) are vectors of several globally important arboviruses, including dengue virus (DENV) (Simmons *et al*.,2012), yellow fever virus (Jentes *et al.*, 2011) and chikungunya virus (CHIKV). The remaining burden of vaccine preventable yellow fever is similarly likely to be dramatically underestimated (Garske *et al.*, 2014). Both infections cause high fevers often accompanied with excruciating joint pain or other flu-like symptoms. The public health impact of DENV and CHIKV has increased dramatically over the last 50 years, with both diseases spreading to new geographic locations and increasing in incidence within their range. Mosquitoes can acquire bacteria transtadially (larvae to adult mosquitoes through bacteria in water) and through sugar-feeding as adults (Pumpuni *et al*., 1996). Almost all of the mosquito-vectors around the globe have successfully learned to defend themselves from the existing insecticides that are being recommended by WHO.

Identifying sustainable microflora for the midgut condition can, therefore, help in evaluating its contribution in mosquito pathogen interaction and in turn vector competence. Recent metagenomic studies of the mosquito midgut have revealed that the presence of a varied bacterial flora has also been suggested to modify the competence of mosquitoes to transmit pathogens (Coon *et al*., 2014; Dillon & Dillon, 2004; Minard *et al*., 2013). The study focuses on the bacterial community in the midgut of mosquitoes by an independent metagenomics approach to culture. Midguts were also used to manipulate the microbiota in the midgut to modulate mosquito vector competence, as a powerful vector management strategy (Yadav et al., 2015) (Wang *et al*., 2012; Hurwitz *et al.*, 2011). Until now, various culture-dependent isolation methods have been used to identify culturable bacteria. Through this study, we would like to explore non-cultivable bacteria using Nonculture based on environmental samples directly for the DNA method. It allows us to identify and determine DNA sequences of species that are difficult to grow in laboratory cultures. In a large-scale metagenomic study (Dinsdale *et al*., 2008). Metagenomics is the culture-independent genomic analysis of microbial communities. The term is derived from the statistical concept of meta-analysis (the process of statistically combining separate analyses) and genomics (the comprehensive analysis of an organism's genetic material) Rondon *et al*., (2000). Metagenomics enables us to study microorganisms by deciphering their genetic information from DNA that is extracted directly from communities of environmental microorganisms, thus bypassing the need for culturing or isolation. This discipline builds on the successes of culture-independent surveys of environmental samples (Olsen *et al*., 1986).

Therefore, the application of tools based on "growing dependent and independent culture", such as 16s rRNA gene sequencing and metagenomics studying these systems is highly desirable. Most of the early results of many studies that have employed microbial diversity independent rRNA 16 culture methods are the wealth of the uncultivated microbial world. The ultimate goal of the research focuses on recent progress in understanding the role of the mosquito midgut microbiota in the modulation of infections by vector-borne pathogens, which could lead to alternative strategies for disease control.

MATERIALS AND METHODS

Mosquito collections and species identification

The laboratory-bred pathogen-free strains of mosquitoes were reared in the vector control laboratory, Department of Zoology, Annamalai University. Mosquitoes were held at 28±2°C, 70-85% relative humidity, with a photoperiod of 12-h light and 12-h dark. The larvae were fed on dog biscuits and yeast powder in the 3:1 ratio. At the time of adult feeding, these mosquitoes were 3-4 days old after emergences

(maintained on raisins and water) and were starved for 12h before feeding. Each time, 500 mosquitoes per cage were fed on blood using a feeding unit filted with parafilm as membrane for 4h. *Ae.aegypti* feeding was done from 12 noon to 4:00 p.m and *Cx. quinquefasciatus* were fed during 6:00 to 10:00 pm. A membrane feeder with the bottom end fitted with parafilm was placed with 2.0 ml of the blood sample (obtained from a slaughter house by collecting in a heparinized vial and stored at 4^{o}C) and kept over a netted cage of mosquitoes. The blood was stirred continuously using an automated stirring device, and a constant temperature of 37 ^{o}C was maintained using a water Jacket circulating system. After feeding, the fully engorged females were separated and maintained on raisins. (Govindarajan M and Sivakumar 2014).

Mosquitoes gut dissection

Mosquitoes were fed with 10% glucose solution before dissection of the midgut. The mosquitoes were surface sterilized with 70% ethanol for 2-10 min, which effectively surface sterilized the mosquitoes but did not affect the midgut bacteria (unpublished data) (Demaio *et al*., 1996). After surface sterilization, mosquitoes were rinsed twice in sterile saline solution (0.85% NaCl). Midguts were dissected and placed individually in 200-µl aliquots of NaCl-solution prepared the day before under sterile conditions. Eppendorf tubes were reclosed immediately after midguts were put in the solution. After each dissection forceps and needles were sterilized thoroughly in 70% ethanol to prevent contamination.

DNA extraction from mosquito Gut

Obtained midguts were stored in -80^{o}C until used for DNA extraction. The first is that DNA must be extracted from the widest possible range of microorganisms to represent the original microbial population. The total microbial DNA was extracted by adapting small changes in the protocol described by Broderick *et al*, (2004). Suspend in 1 l of digestion buffer. Incubate samples with occasional shaking in micro tubes hermetically sealed for 1 hour at 37 ° C. Centrifuge at 13,000 rpm for 30 minutes at -4ºC. The supernatant was transferred to some centrifuge tubes. The samples were extracted with an equivalent phenolic centrifuge: chloroform: isoamyl alcohol 25:24:1 at 13000 rpm for 15 minutes at -4°C. The supernatant was transferred to 1.5 µl of hemmomelic tube. Incubate at -80 ° C for 30 minutes. Centrifuge at 13,000 rpm for 30 minutes at 4°C. Reveal the supernatant of Tesnapend in 20 ml of TE buffer (ph -> 8.0) store at -20 °C and use more.

DNA quality and quantity

The absorption spectrum of DNA extracts (230–280 nm and 260-230nm) was determined using Nano-drop(R) ND-1000 spectrophotometer (Eurofins Genomics Bioinformatics Lab) according to the manufacturer's instructions. Pure DNA is known to produce 260/280 and 260/280nm ratios 1.80 (Maniatis *et al*., 1982). DNA was visualized by electrophoresis of 5-µl aliquots through 1.2% (w V-1) agarose gel containing 0.5 µg ml−1 ethidium bromide, and DNA was quantified (µg DNA 0.1g−1 fresh gut content) as previously described.

Polymerase chain reaction (PCR) amplification

The PCR reaction of bacterial 16S rRNA gene V3 and V4 region was performed containing 12.5 µl of 2x KAPA HiFi HotStart ReadyMix, 5 µl (1 µM) of forward primer GCCTACGGGNGGCWGCAG 3' and reverse primer 5' ACTACHVGGGTATCTAATCC 3' and 2.5 µl of DNA template (5 ng/µl in 10 mM Tris pH 8.5) to a final volume of 25 µl. The PCR protocol was performed in triplicate using the following conditions: 5 min at 98 °C for initial denaturing, followed by 25 cycles of 98°C for 30 s, 50°C for 30 s and 72°C for 30 s with the final extension for 5 min at 72 °C. The Illumina sequencing adapter ligated reverse primer contained a 6-bp barcode specific for sample identification (Caporaso *et al*., 2012). After amplification, PCR products were pooled and purified using the PCR cleanup Kit (Axygen Biosciences, Union City, CA, USA). Bacterial PCR products were pooled separately to sequence in their runs, respectively.

Cluster generation and Sequencing

After obtaining the peak size from Tape Station profile, libraries were loaded onto MiSeq at an appropriate concentration (10-20pM) for cluster generation and sequencing. Paired-End sequencing allows the template fragments to be sequences in both the forward and reverse direction on MiSeq. The kit reagents were used in the binding of samples to complementary adapter oligos on paired-end flow cell. The adapters were designed to allow selective cleavage of the forward strands after re-synthesis of the reverse strand during sequencing. The copied reverse strand was then used to sequence from the opposite end of the fragment.

Bioinformatics and statistical analysis

Bacterial sequences were analyzed using the UPARSE pipeline. Briefly, paired end reads were merged into single sequences, the low-quality merged sequences

(maximum expected error higher than 1, shorter than 370 bp for bacterial) were removed from downstream analysis. After removing the chimera, sequences with ≥97% similarity were clustered into Operational Taxonomic Units (OTUs). The OTU representative sequences were assigned using the RDP classifier to identify bacterial taxonomies with a confidence threshold of 0.5 (Caporaso *et al*., 2012).

RESULTS

NanoDrop reading and station profile of gDNA

The nanoDrop readings showed that the quantity and quality id DNA, the readings showed that the quantity of 267.6 ng/µ1 (table 1).

Table1: Showing the Nanodrop reading of the sample

Sample ID	NanoDrop Readings (ng/µ1)	NanoDrop ODA$_{260/280}$	NanoDrop ODA$_{260/230}$	Remark
Aedes	267.6	1.93	1.78	QC Pass

Tape- station profile of libraries of sample

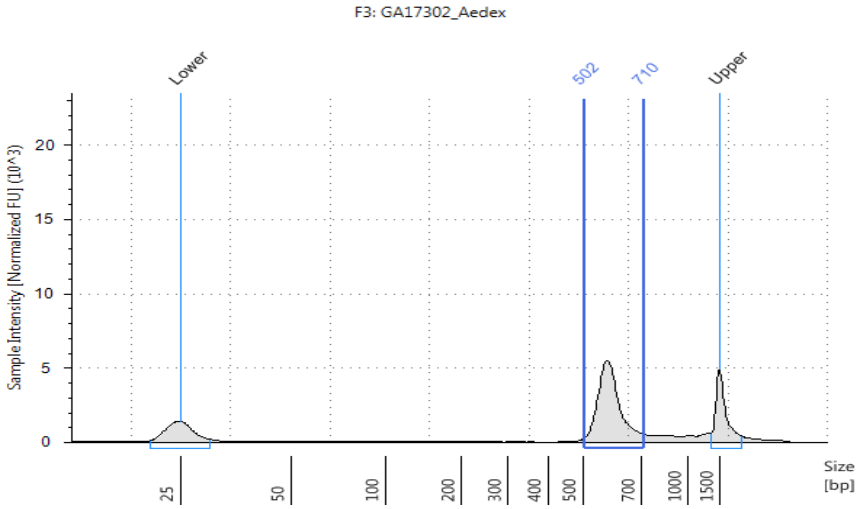

Figure 1: Library profile of sample of *Aedes* on Agilent tape station using D1000 screen tape

QC pass DNA sample were processed for first amplicon generation followed by NGS library preparation using Nextera XT Index Kit (illumine Inc.). The mean of the library fragment size distribution is 596bp for sample *aedes*. Libraries were sequence of MiSeq using 2×300 bp chemistry (figure 1).

Table 2: showing the total bases read in the samples

Inputs	Reads	Bases	Avg-Len
Aedex_R1. Fastq	89656	24912436	27787
Aedex_R2. Fastq	89656	21481009	239.59

Bacterial Diversity in mid gut of *Aedes aegypti*

The total reads counts were found to be 89656 in *A. aegypti* gut part. Metagenomic analysis of gut sample from *A. aegypti* showed the presence of different bacterial community (table 2) similarly was reported that the earthworm mid gut has showed verity of bacterial genera was found. After tag extraction and filtering of low-quality sequence tags, we obtained 179,310 reads for the analysis, representing 99.9% of the total reads. OUT diversity in the whole population was high with the detection of 209 different bacterial genera distributed in twenty phyla (Figure 2). Among which *Proteobacteria, Firmicutes, Bacteroidetes* and *Actinobacteria* were found to be of high abundance.

Planctomycetes, Chloroflexi, Cyanobacteria, Deinococcus-Thermus, Acedobacteria, Fusobacteri and *Verrucomicrobia* were found to be in negligible amount. *Proteobacteria* largely dominated the midgutmicrobiota of *Aedes* mosquitoes. Their diversity encompasses *Alpha, Beta* and *Gamma* super classes of *proteobacteria*. Figure 3 displays the Krona chart showing the bacterial diversity of *A. aegypti*. This analysis showed that majority of the gut bacterial community corresponded to members of the *Gammaproteobacteria* class with most assignments being to members of the *Enterobacteriaceae, Aeromonadacea e, Flavo bacteriaceae* and *Moraxellaceae* families. The diversity was lower in *alpha-* and *beta-proteobacteria*. *Burkholderiales*, which belonged to *Beta-proteobacteria* and *Rhizobiales*, *Sphingomonadales* belonging to *alpha-proteobacteria,* were also found at lower range. Other families with remarkably high representation in the gut were *Comamonadaceae*, *alteromonadaceae, Bacillaceae, Staphylococcaceae, Pseudomonadaceae* and *Rhodobacteraceae*.

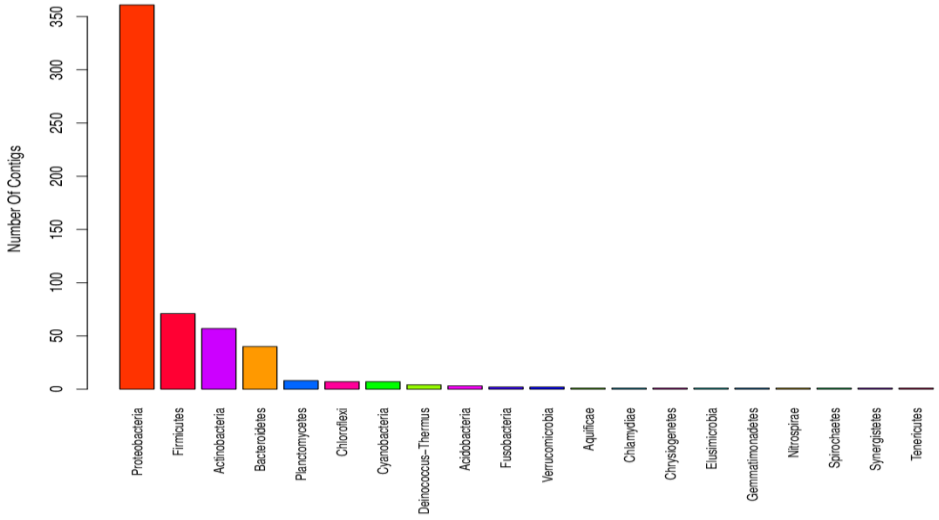

Figure 2: Number of reads represented by 20 phyla detected in *Aedes* gut samples *Proteobacteria* occupies high abundance. *Aguificae, Chlamydiae, Chrysiogenetes, Elusimicrobia, Gemmatimonadetes, Nitrospirae, Spirochaetes* and *Tenericutes* were represented by few reads.

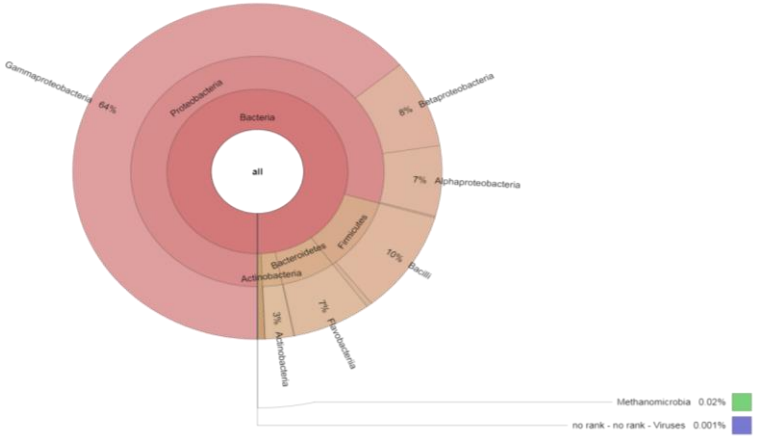

Figure 3: Krona chart showing bacterial diversity for *Aedesagypti*

The figure 3 depicts the abundance existence of *Proteobacteria* especially *Gammaproteobacteria* in the gut of mosquito. Presence of other phyla members including *Firmicutes, Bacteroidetes* and *Actinobacteria*were also found. Only a negligible amount of*actinobacteria, acidobacteria, cyanobacteria* and *ignavibacteria* were observed. The reads were classified into 209 genera of which the most abundant genera occupied by gut microbiome were *Aeromonas, Klebsella, Acinetobacter, Staphylococcus, Bacillus,*

Pseudomonas, Shewanella, Corynebacterium, Alteromonas, Enterobacter, Methylobacterium and Riemerella. Escherichia, Flavobacterium, salmonella, Agrobacterium, Thallasolittus, vibrio, Erwina, Erythrobacter, Anaerococcus and Paenibacillus were observed at lower abundance.

Bacterial Taxonomic distribution at species level

Edge uses both read based and contig based classification along with BWA, a read mapper used against RefSeq. A total of 284 species-level taxa were detected in the samples. The Krona chart showing the species level distribution of microbiome is depicted in figure 4. *Aeromonasveronii, Klebsiellamichiganensis, Staphylococcus saprophyticus, Flavobacteriaceae bacterium 3519-10, Klebsiella pneumonia, Riemerellaanatipestifer, Acinetobacter baumannii, Sphingomonaswittichii, Aeromonashydrophilia* and *Variovoraxparadoxus* were the most abundant. The heat map showing the species level bacterial distribution is displayed in figure 4.

DISCUSSION

The present study was undertaken to study the bacterial diversity cultural and uncultivable of the selected to mosquito species *Aedes agypti* gut sample using Illumina Miseq studies. The mosquito species *aedes agypti* for the supplied mosquito gut samples by using phenol: chloroform Extraction method. The absorption spectrum of DNA extracts (260-280nm and 260-230nm) was determined using Nano-drop(R) ND-1000 spectrophotometer (Eurofins Genomics Bioinformatics Lab) according to the manufactory's instructions. Sample *Aedesagypti* showed nanodrop reading for 1.93 and 1.78 for 260-280nm and 260-230nm nanodrop reading respectively. The amplicon libraries were prepared using Nextera XT Index (Illuminainc). As per the 16s metagenomic sequencing library preparation protocol primers for the amplication of the 16s rDNA gene specific for bacteria were designed at Eurofins Genomics Bioinformatics Lab and Synthesized at Eurofins Genomic Lab facility. The total bases read of bacterial diversity for the mosquito *Aedes agypti* species.

Figure 4: Taxonomic evaluations of the bacterial isolates. (A) Krona chart showing species level bacterial diversity for *Aedesagypti.* This shows the species level abundance of various bacteria. (B) Read-based classification using various taxonomy profiling tools.

In the present study it is seen that *proteobacteria* is predominant with highest count in mosquito *aedes agypti*. Mosquito-borne diseases menace the living and livelihood of millions of people worldwide (Tamizhazhagan *et al*., 2017). In total, *Gammaproteo bacteria* accounted for the vast majority of the community metagenome, with 64% of the total *proteobacteria*.*Firmicutes, Bacteroidetes* and *Actinobacteria*were also found to be of high abundance. These bacterial phyla are generally reported in the guts of mosquitoes and other insects (Boissière *et al*., 2012; Engel & Moran, 2013; Osei-Poku *et al*., 2012). Fertilizers can directly stimulate the growth of microbial populations as whole by provision nutrients and should have an effect on the composition of individual microbial communities within the soil (Tamizhazhagan *et al*., 2016).

It was reported that some midgut dwelling bacteria play an important role in disease transmission and host-parasite interaction and affects the vectorial capability of mosquitoes. The midgut serves as the first contact point between parasites and the epithelial surfaces, where significant parasite numbers are reduced. The microbiota involved in the blocking of the Plasmodium development may be used in the modulation of vectorial capacity of mosquitoes (Dong *et al*., 2009). Midgut microbiota is recognized to modulatethe immune response of the mosquito and influence vector competence to human pathogens. Some bacteria may act directly by producing molecules with antiviral activity. Others may indirectly interfere by activating signaling pathways that stimulate the immune system of insect to fight with the invader (Dong *et al.*, 2009). Since immune competent mosquitoes are thought to be less likely to transmit other parasites such as malaria (Arbain, 1990), similar strategies might also be supportive in controlling denguewith the use of bacterial species that enhance the mosquito's immune system. The tripartite relationship between mosquito, its microbiome and human pathogens is critical in disease transmission. The midgut microbiota composition has a significant role on the vulnerability of chikungunya and dengue viruses. It has been proved that the vulnerability of *A. aegypti* to chikungunya and dengue virus increases in the presence of midgut bacteria *Serratia odorifera* due to the suppression *A. Aegypti* immune response (Apte *et al*., 2014). *A. aegypti* fed with the *Aeromonas sp*. and *Escherichia coli* showed elevated susceptibility to DENV-2 (Rani *et al*, 2009)

It is clear that the midgut microbes can be significantly engaged in host-parasite interaction and may decrease or increase the vectorial capacity through various mechanisms including improvement of immune response or precluding the development of parasites. Midgut microbiota may be genetically manipulated to express molecules against the parasites, which could be used as a novel strategy for vector management. The understanding of midgut microbiota and the mosquitoes could be utilized for the development of novel, eco-friendly and highly effective defense mechanism to reduce the vectorial capacity of mosquitoes and consequent control of disease transmission. As the microbiota might have an impact on pathogen development in *aedes* mosquito and disease transmission, more studies need to be done for better understanding the role of some specific bacteria in valid mosquito population before developing potential method of control.

CONCLUSION

The identification of the sustainable microflora of the midgut environment can therefore help in evaluating its contribution in mosquito pathogen interaction and in turn vector competence and to understand the bacterial community in the midgut of mosquito. Further studies are needed to investigate by physiological characteristics of the bacterial isolates and their possible interactions with mosquito by biology and vector competence.

Acknowledgement

The author would like to thank Dr. K. Yoganathan, Dr. David Rajendran and Dr. Beutline Malgija for their timely help during the manuscript preparation and submission. Also, we would like to extent our thanks to Entomology Research Institute of Loyola College, Chennai and Yaazh Xenomics laboratories giving excellent technical and bioinformatics support.

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Received:05.08.18, Accepted: 08.09.18, Published:01.10.2018

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