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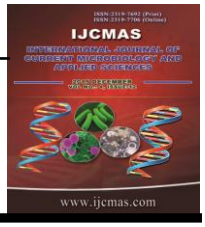

Original Research Article

Studies on Mid Gut Microbiota of Wild Caught Aedes (*Aedes albopictus*) Mosquitoes from Diamond Harbour (South 24 Parganas) Areas of West Bengal, India

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A B S T R A C T

Mosquito midgut microbiota plays a crucial role in its vector susceptibility and pathogenic or parasitic interaction. To understand the bacterial diversity in the midgut of *Aedes albopictus* mosquitoes, we conducted a screening study of the gut microbes of these mosquitoes which were collected from fields and reared in the laboratory. Mosquitoes are haematophagous vector and carries parasites and pathogens of numerous diseases like Dengue, Filaria, Malaria, etc. Mosquitoes can be considered as holobiont units in which host (mosquitoes) and its gut microbiota are involved in a complex reciprocal interaction. The naturally acquired microbiota can modulate mosquitoes' vectorial capacity by inhibiting the development of pathogens and parasites. But enough care has not been taken in West Bengal to study on midgut microbiota of *Aedes* mosquitoes. Therefore a preliminary attempt has been undertaken to study the mid gut microbiota of *Aedes* (*Aedes albopictus)* mosquitoes collected from Diamond Harbour (South 24 Parganas) areas of west Bengal.

K e y w o r d s

Aedes albopictus, Gut microbiota, Antibiotic susceptibility

Introduction

Symbiotic micro organisms play an important role in the biology of vector species (mosquito). Several scientists (Moran et. al, 2008; Moya et.al, 2008) reported that the vectors are known to harbor a number of commensal and mutualistic micro organisms that have an impact on the ecology and ethology of host. Mosquito can be considered as an holobiont units in which the host (mosquito) and its microbiota are involved in complex reciprocal multipartite interactions (Rosenberg et al, 2011). The present global research on vector and vector borne diseases have been extended for using the microorganism as biological control agent (Beard et.al 2002). Microorganism

associated with vectors could exert a direct pathogenic effect on the host (Schnepf et.al 1998), interfere with its reproduction or reduce vector competence(Zabalou et.al, 2004).This symbiosis provides an important physiological functions to the host, including the synthesis of essential nutrients, resistance to colonize pathogens and stimulate the immune system (Dillon et al, 2005, Dong et al,2009). In hematophagous insects these symbionts are thought to be critical for the host fitness because of the need for blood scarce nutrients. The mosquito mid gut is an immune competent organ and the first point of contact between the vector and arboviruses. Therefore, the microbial community structure of the mosquito mid gut may play a crucial role to identify parasite or pathogen (virus) entry, multiplication and in turn, vector susceptibility. Recent studies have been forwarded for detecting the diverse microbial community in this physiological niche (Dong et al,2009, Jadin et al, 1966, Seitz et al, 1987, Pumpuni et al,1996, Straif et al, 1998, Gonzalez et al, 2003, Lindh et al, 2005, Favia et al, 2007, Gusamo et al, 2010) . These studies not only emphasizes on mid gut microbial diversity but also with its morphology and biochemical characteristics. Mosquitoes, are the known group of haematophagous vector, from the stand point of both basic and applied science (Banerjee 1997) *Ae. albopictus* is a natural vector for a group of viruses such as chikungunya, dengue fever and yellow fever. In spite of its epidemiological importance in disease transmission, very little informations are available on *Aedes* mosquito with respect to its gut microflora and its interaction with the disease transmitting agent. Identification of the stable and transmissible gut inhabitant of such a vector would therefore be a crucial factor in understanding its susceptibility to a battery of viruses and parasites. It has been reported (Das P.K,1997) that the occurrence of vector borne diseases in any place or at any time is determined by the complex interaction of pathogen, parasite, microorganisms and vectors in a particular environment. Various lines of data (Chernysh et al,2002; Wilkinson et al,2001; Zhang et al,2004) indicated that these diverse microbiota are a potential source of novel bioactive compounds viz. anti-viral, anti-malarial, anti-tumor peptides, enzymes and novel metabolites. Manipulating these microbial symbionts is thought to be an effective strategy for controlling the spread of pathogens that use insects (mosquitoes) as hosts (Beard et al, 2002; Dillon et al, 2005;).*Aedes albopictus*, an anthropophilic mosquitoe is not only responsible for the transmission of dengue viruses, but also acts as a reservoir of a large variety of gut microbes .Recent resurgence of dengue and dengue hemorrhagic fever in Kolkata and adjoining sub urban areas have necessitated to study on *Aedes albopictus.* But a very little attention has been paid to know the morphology and biochemical characteristics of mid gut microbiota of *Aedes albopictus* in the various regions of West Bengal. In view of these reasons a preliminary attempt has been made to find out morphological and biochemical characteristics of midgut microbiota of *Aedes albopictus* mosquitoes in Kolkata and allied areas like Diamond Harbour (South 24 Parganas) of west Bengal.

Materials and Methods

Collection of Mosquitoes

*Aedes albopictus m*osquitoes were ariely collected during early morning (5.30 a.m. to 6.30 a.m.) for a period (from June, 2014 to June,2015) from Diamond Harbour, district of South 24 Parganas of West Bengal (Latitude: 22.2°N, Longitude: 88.2°E) (fig1)

using manual aspirator. The temperature and relative humidity range during the time period were (25°C to 37°C) and (50% to 90%) respectively. The collected samples were transported to the laboratory in transfer glass bottles with perforated cap to keep them alive. Only live mosquitoes were selected for microbiological analysis.

Colonization

Mosquito samples were killed with chloroform (90%v/v) followed by surface sterilize using 70% ethyl alcohol(v/v). Guts of the samples were removed using sterile forceps and then mixed thoroughly with 500µl of sterile physiological saline [0.7% (w/v) aqueous solution of sodium chloride, pH 7.2,at 25°C] on a sterile enclosed glass crucible. The midgut suspension was then streaked on sterile nutrient agar (Peptic digest of animal tissue 5.000 Gms / Litre, Sodium chloride 5.000 Gms / Litre, Beef extract 1.500 Gms / Litre ,Yeast extract 1.500 Gms / Litre ,Agar 15.000 Gms / Litre, Final pH (at 25°C) 7.4±0.2) plates. Triplicate plates were then incubated at (30±2)°C for (24+24) hours. Isolated colonies were selected based on their morphotyping, subcultured as axenic cultures on nutrient agar plates and stored as slants at 4°C for future characterization.

Gram staining of Microorganisms

Gram staining of each culture was carried out following the procedure of Harrigan and MacCance (1976)

Standard Biotyping Assays

The standard Biotyping assays were performed to identify the common growth and metabolic patterns of each of the bacterial isolates.

Amylase Production Detection Assay

Overnight (18-20 hrs.) Nutrient broth culture suspensions of the isolates were prepared (as mentioned earlier). Sterile Starch agar medium (Starch soluble, 20gms/ltr.; Peptone, 5gms/ltr; Beef extract, 3gms/ltr.; Agar, 15gms/ltr.; Final pH at 25^0C 7.0 ± 0.2) were prepared and cast on sterile disposable petri plates. The isolate suspension was streaked onto the Starch agar plates using standard 4 way discontinuous streaking protocol and incubated at 35^oC ± 2^oC for 18 to 48 hours. After incubation the growth colonies on the plates were scraped off using a sterile inoculation loop and the plates were flooded with a dilute iodine solution for 60 seconds. Excess iodine drained off. Results were observed from a study of triplicates.

Protease Production Detection Assay

Overnight (18-20 hrs.) Nutrient broth culture suspensions of the isolates were prepared (as mentioned earlier). Sterile Standard count Caseinate agar medium (Casein enzymic hydrolysate 5 gms/ltr.; Yeast extract 2.5 gms/ltr.; Dextrose 1 gm/ltr.; Sodium caseinate 10 gms/ltr.;

Trisodium citrate 10 gms/ltr.; Calcium chloride 2.2gms/ltr.; Agar 15 gms/ltr.; Final pH at 25^0C 7.2 ±0.2) were prepared and cast on sterile disposable petri plates. The isolate suspension was streaked onto the medium plates using standard 4 way streaking protocol of Loeffler and Gaffky(1881) and incubated at 35^oC ± 2^oC for 18 to 48 hours. Results were observed.

DNase Production Detection Assay

Overnight (18-20 hrs.) Nutrient broth culture suspensions of the isolates were prepared (as mentioned earlier). Sterile DNase Test Agar w/ Toluidine blue medium (Tryptose 20 gms/ltr.; DNA powder 2 gms/ltr.; NaCl 5 gms/ltr.; Toluidine blue 0.1 gm/ltr.; Agar 15

gms/ltr.; Final pH at 25^{0}C 7.2 ± 0.2) were prepared and cast on sterile disposable petri plates. The isolate suspension was streaked onto the medium plates using standard 4 way streaking protocol of Loeffler and Gaffky(1881) and incubated at 35^{o}C ± 2^{o}C for 18 to 48 hours. Results were observed.

Gelatin Solubilization Activity Detection Assay

Overnight (18-20 hrs.) Nutrient broth culture suspensions of the isolates were prepared (as mentioned earlier). Sterile Gelatin iron agar medium (Peptic digest of animal tissue 25 gms/ltr.; Meat extract 7.5 gms/ltr.; NaCl 5 gms/ltr.; Gelatin 120 gms/ltr.; Ferrous chloride 0.5 gms/ltr.; Final pH at 25^{0}C 7.0 +/- 0.2) were prepared and cast as stabs in sterile culture tubes. The isolate suspension was pierced into stab with the help of a sterile inoculation needle and incubated at 35°C ± 2^{o}C for 18 to 48 hours. Results were observed.

Arginine Dihydrolase Production Detection Assay

Overnight (18-20 hrs.) Nutrient broth culture suspensions of the isolates were prepared (as mentioned earlier). Sterile Arginine Dihydrolase agar medium (Peptic digest of animal tissue 1gm/ltr.; NaCl 5 gms/ltr.; Dipotassium hydrogen phosphate 0.3 gm/ltr.; L-Arginine 10 gms/ltr.; Bromo cresol purple 0.016 gm/ltr.; Agar 3 gms/ltr.; Final pH at 25^{0}C 6.0 ± 0.2) were prepared and cast as stabs in sterile culture tubes. The isolate suspension was pierced into stab with the help of a sterile inoculation needle and incubated at 35^{o}C ± 2^{o}C for 18 to 48 hours. Then 5-6 drops (600 µl-800 µl) of the Nessler's Reagent [Mercuric iodide 3%, Potassium Iodide 3.5%, Sodium Hydroxide 12%, and Water 81.5%] was added to the culture tube. Results in colour change were observed.

Catalase Production Detection Assay

Overnight (18-20 hrs.) Nutrient broth culture suspensions of the isolates were prepared (as mentioned earlier). Sterile Nutrient agar medium were prepared and cast on sterile disposable petri plates. The isolate suspension was streaked onto the medium plates using standard 4 way discontinuous streaking protocol and incubated at 35^{o}C ± 2^{o}C for up to 48 hours. A few drops (500 µl-600 µl) of 15% H_2O_2 were poured over the grown colony and observed immediately for effervescent bubble production.

Oxidase Production Detection Assay

Overnight (18-20 hrs) Nutrient broth culture suspensions of the isolates were prepared (as mentioned earlier). Sterile Nutrient agar medium were prepared and cast on sterile disposable petri plates. The isolate suspension was streaked onto the medium plates using standard 4 way discontinuous streaking protocol and incubated at 35^{o}C±2^{o}C for up to 48 hours. The test was performed by putting bacterial culture on a strip of sterile filter paper impregnated with 1% (w/v) aqueous solution of N-N-dimethyl-phenylenediamine. Immediate colour change was observed.

Endo Agar Assay

Overnight (18-20 hrs) Nutrient broth culture suspensions of the isolates were prepared (as mentioned earlier). Sterile Endo agar base medium (Peptic digest of animal tissue 10.000 gms/ltr, Lactose 10.000 gms/ltr , Dipotassium phosphate 3.500 gms/ltr, Sodium sulphite 2.500 gms/ltr, Agar 12.000 gms/ltr, Final pH (at 25°C) 7.5±0.2) were prepared and cast on sterile disposable petri

plates. The isolate suspension was streaked onto the medium plates using standard 4 way streaking protocol of Loeffler and Gaffky(1881) and incubated at 35°C ± 2°C for 18 to 48 hours. Results were observed.

King's B assay

Overnight (18-20 hrs.) Nutrient broth culture suspensions of the isolates were prepared (as mentioned earlier). Sterile King's medium B Base medium (Proteose peptone 20.000 gms/ltr
Dipotassium hydrogen phosphate 1.500 gms/ltr, Magnesium sulphate. heptahydrate 1.500 gms/ltr , Agar 20.000 gms/ltr, Final pH (at 25°C) 7.2±0.2) were prepared and cast on sterile disposable petri plates. The isolate suspension was streaked onto the medium plates using standard 4 way streaking protocol of Loeffler and Gaffky(1881) and incubated at 35°C ±2°C for 18 to 48 hours. Results were observed.

Antibiotic Sensitivity Assay

The bacterial isolates were tested for their antibiotic resistance pattern against nine commercially available antibiotics. Antibiotic impregnated paper discs (HiMedia) used for the study include amikacin (30µg $disc^{-1}$), ciprofloxacin (5µg $disc^{-1}$), colistin (10µg $disc^{-1}$), gentamicin (10µg $disc^{-1}$), imipenem (10µg $disc^{-1}$), netillin (30µg $disc^{-1}$), polymyxin-B (300µg $disc^{-1}$), and ticarcillin (75µg $disc^{-1}$). Isolated bacterial colonies of each isolates were grown in Muller Hinton broth for 6hours at (30±2)°C at 110 rpm shaking, followed by subsequent preparation of an inoculums lawn on Muellar-Hinton Agar (Beef, infusion from 300.000 Gms / Litre, Casein acid hydrolysate 17.500 Gms / Litre, Starch 1.500 Gms / Litre , Agar 17.000 Gms / Litre, Final pH (at 25°C) 7.3±0.1) plates using cotton swab. The selected antibiotic discs were placed aseptically using sterile forceps keeping a distance of 4cm between their centres. Complete inhibition zone around each disc was measured after 18hours of incubation at (30±2)°C [1]. Each experiment was performed in triplicate.

Results and Discussion

Bacterial Isolates

Four morphotype of bacterial isolates were selected from samples of mosquito. The characteristics of each morphotype and the number of isolates studied from each morphotype are presented in Table-1.

Gram Characteristics of Microorganism

All morphotype isolates showed positive Gram reaction. Gram character, morphology and arrangement of cell in each morphotype are shown in Table-2.

Standard Biotyping Assay

Standard biotyping assays of each of bacterial isolates are shown in Table-3

Antibiotic Susceptibility

Analysis of isolates with lowest and highest resistance patterns are shown in table4 and figure2.

Our findings provide comprehensive information about colony character , gram staining properties and biochemical characteristics of mid gut microbiota of *Aedes albopictus* mosquitoes collected from Diamond Harbour areas (Fig1) of west Bengal. Our data (Table-1 and 2) reveal that the gut-bacterial colonies are more or less circular, coccus shapped, arranged in singly or in paired and umbonate shape and gram positive.

Table.1 Morphotypes of Isolated Colony

Sample	Morpho type	Morphological Characters of colony						No. of isolates selected
		shape	Size	colour	Visibility and texture	elevation	type	
Aa	M1	circular	2-3mm in diameter	brown	Opaque, matte	plain	Diffuse, margin entire	2
Aa	M2	circular,	2mm in diameter,	yellow	Opaque, matte	convex	Diffuse, margin entire	3
Aa	M3	circular	2-3mm in diameter	whitish	Opaque, matte	plain	Diffuse, margin entire	2
Aa	M4	umbonate	2-3mm in diameter	creamish	Opaque, glistening	plain	Diffuse, margin entire	1

Table.2 Gram Character, Morphology and Arrangement of Cells in each Morphotype

Isolates from morphotype	Gram Character	Morphology and arrangement
AaM1	Gram Positive	Coccus shaped, mostly in chains.
AaM2	Gram Positive	Coccus shaped, mostly in pair.
AaM3	Gram Positive	Coccus shaped, mostly in pair.
AaM4	Gram Positive	Coccus shaped, clumped, held together.

Table.3 Standard Biotyping Assays of each of Bacterial Isolates

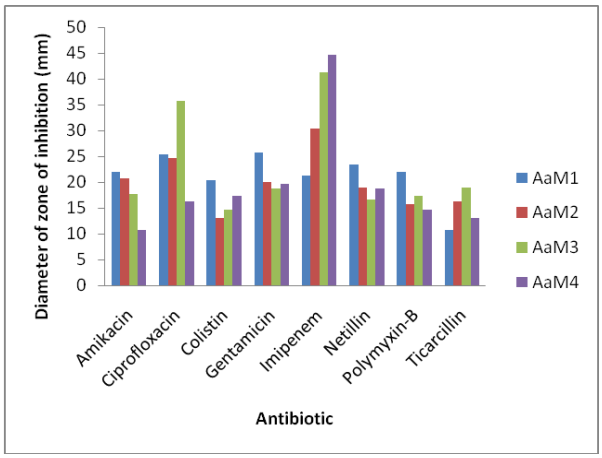
Sample No	Catalase production	Oxidase production	DNase production	Gelatin production	Arginine dihydrolase production	King's B assay	Endo agar assay	Amylase Production assay	Protease Production assay
AaM1	-VE	+VE	-VE	-VE	-VE	+VE	+VE	-VE	+VE
AaM2	-VE	-VE	-VE	-VE	-VE	-VE	+VE	-VE	+VE
AaM3	+VE	-VE	-VE	-VE	-VE	+VE	+VE	-VE	+VE
AaM4	-VE	-VE	+VE	-VE	-VE	+VE	+VE	-VE	+VE

Table.4 Assessment ofAntibiotic Sensitivity Assay
Antibiotic Sensitivity Patterns (Resistant (R) / Sensitive(S)) as per CLSI standards

Name of the Isolates	Ticarcillin	Gentamicin	Imipenem	Ciprofloxacin	Polymyxin-B	Colistin	Netillin	Amikacin
AaM1	R	S	S	S	S	S	S	S
AaM2	S	S	S	S	S	S	S	S
AaM3	S	S	S	S	S	S	S	S
AaM4	R	S	S	S	S	S	S	R

Figure.1 Map of South 24 Parganas Showing the Collection Site-Diamond Harbour (www.mapsofindia.com)

Figure.2 Antibiogram of Mean zone Inhibition of gut Bacterial Isolates from each Morphotype of Five Sample against Nine Antibiotics



These bacterial isolates demonstrate their unique property of secreting economically important enzymes. The different biochemical tests (Table- 3) reveal that protease, DNase, oxidase, catalase and gelatin are produced by one isolates. On the other hand, protease are secreted by all bacterial isolates. These biochemical properties of secretion of novel enzymes provide these bacterial isolates has the

potential activity for commercial approach. As for example, protease producing bacteria has the potential for degrading proteinaceous wastes. Antibiotic susceptibility tests (Table-4) indicated that midgut microbiota of *Aedes albopictus* are found to be moderately resistant to the antibiotics such as Ticarcillin and Amikacin and more or less sensitive against all other antibiotics (fig2) used. Mosquitoes are known to illicit a specific immune response against parasite and bacteria (Gram positive and Gram negative). Several lines of data **(**Kasai et al, 1986) indicates that immune activity inhibit the growth of pathogens viz *chikungunya, dengue fever virus, yellow fever virus as well as protozoan parasite (Plasmodium gallinaceum)* etc. It can suggest that this microbiota may also illicit basal immunity to the vector. Mosquitoes (*Aedes*, *Anopheles e*tc), usually live in highly contrasting environments where biotic (like competition or the food chain) and abiotic (like temperature or humidity) factors can influence the population of gut microbiota. Observation of biochemical properties may help to know the diversity of mid gut micribiota. Mid gut microbes are closely associated with vector (mosquitoes) through a complex potential interaction. Symbiotic interaction between the mid gut bacteria (*Asia*) has potential power in blocking transmission of mosquito borne pathogens through paratransgenesis (Favia et.al 2007). Current technologies are not sufficient to pin point all the fluxes of matter and energy between microorganism and their host. However, a little investigation has been highlighted on the beneficial functions of mid gut bacteria, especially those living intracellularly as endosymbionts **(**Moffatt et al 2010**)**. It can be concluded that the study on midgut microbiota along with their different biochemical characteristics may open new windows for better understanding.

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