



Original Research Article

Studies on Mid Gut microbiota of wild caught *Culex (Culex quinquefasciatus)* mosquitoes from Diamond Harbour (South 24 Parganas) areas of West Bengal

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ABSTRACT

Keywords

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Mosquitoes are haematophagous vector and carries parasites and pathogens of numerous diseases like Filariasis, Malaria, Dengue 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. But enough care has not been taken in West Bengal to study on midgut microbiota of *Culex* mosquitoes. Therefore a preliminary attempt has been undertaken to study the morphology, and antibiotic susceptibility of midgut microbiota of (*Culex quinquefasciatus*) mosquitoes collected from Diamond Harbour (South 24 Parganas) areas of west Bengal.

Introduction

Mosquitoes, the known group of haematophagous vector, from the standpoint of both basic and applied research (Mourya et al, 2002). *Culex quinquefasciatus* is the principal vector of filariasis and Japanese Encephalitis (JE) in India. According to various data (Das P.K, 1997) reported that the occurrence of vector borne diseases in any place or at any time is determined by the complex interaction of host, parasite, microorganisms and vectors in a particular environment. All insect species specially the vectors are known to harbor a rich and complex community of microorganisms in their guts. 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). 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-malarial, anti-viral, 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 as hosts (Beard et al, 2002; Dillon et al, 2005; Lehane et al, 1997). *Culex*

Culex quinquefasciatus, an anthropophilic mosquito, is not only responsible for the transmission of filaria and Japanese Encephalitis (JE) but also acts as a reservoir of a large variety of gut microbes. But a very little attention has been paid to know the gut bacterial interaction in *Culex* mosquitoes in the various regions of West Bengal. In view of these reasons we have undertaken a preliminary investigation has been made during the year 2013-2014 to find out morphological characteristics such as Gram staining, pattern of microbial growth and antibiotic susceptibility assay of mid gut bacterial isolates of *Culex* (*Culex quinquefasciatus*) mosquitoes in Diamond Harbour areas (South 24 Parganas) of west Bengal.

Materials and Methods

Collection of mosquitoes

Culex quinquefasciatus mosquitoes were ariely collected during early morning (5.30 a.m. to 6.30 a.m.) everyday for a study period of 12 months (from January, 2014 to December,2014) 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°C 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°C ± 2°C 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 25⁰C 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 35⁰C ± 2⁰C 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 25⁰C 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 35⁰C ± 2⁰C 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 25⁰C 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 35⁰C ± 2⁰C 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 25⁰C 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 35⁰C ± 2⁰C 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 35⁰C ± 2⁰C for up to 48 hours. A few drops (500 µl-600 µl) of 15% H₂O₂ 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 $35^{\circ}\text{C}\pm 2^{\circ}\text{C}$ 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 $35^{\circ}\text{C} \pm 2^{\circ}\text{C}$ 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 $35^{\circ}\text{C} \pm 2^{\circ}\text{C}$ 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 ($30\mu\text{g disc}^{-1}$), ciprofloxacin ($5\mu\text{g disc}^{-1}$), colistin ($10\mu\text{g disc}^{-1}$), gentamicin ($10\mu\text{g disc}^{-1}$), imipenem ($10\mu\text{g disc}^{-1}$), netillin ($30\mu\text{g disc}^{-1}$), polymyxin-B ($300\mu\text{g disc}^{-1}$), tetracycline ($30\mu\text{g disc}^{-1}$) and ticarcillin ($75\mu\text{g disc}^{-1}$). Isolated bacterial colonies of each isolates were grown in Muller Hinton broth for 6hours at $(30\pm 2)^{\circ}\text{C}$ at 110 rpm shaking, followed by subsequent preparation of an inoculum lawn on Mueller-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 $(30\pm 2)^{\circ}\text{C}$ [1]. Each experiment was performed in triplicate.

Results and Discussion

Bacterial isolates

One morphotype each of bacterial isolates were selected from four samples of mosquito (C1-C4) while two were selected from the other sample(C5). The

characteristics of each morphotype and the number of isolates studied from each morphotype are presented in Table-1.

Gram characteristics of microorganism

Out of the six morphotypes tested from five samples, 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 figure3.

Our findings provide comprehensive information about colony character, gram staining properties and biochemical characteristics of mid gut microbiota of *Culex* mosquitoes collected from Diamond Harbour areas (South 24 Parganas) of West Bengal. Our data (Table-1) reveal that the gut-bacterial colonies of Diamond Harbour areas are more or less circular and umbonate shape. Observation from Gram staining (table-2) indicated that bacterial isolates from Diamond Harbour areas are gram positive, coccus shaped, arranged in singly or in pair. 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, arginine dihydrolase are produced by two bacterial isolates. However, amylase are secreted by all bacterial isolates. These biochemical properties to secrete novel

enzymes project these bacterial isolates as potential candidates for commercial importance. As for example, amylase producing bacteria can be used for production of amylase immobilized in beads and are used in detergents to degrade starch stains in cloths. Protease producing bacteria has the potential for degrading proteinaceous wastes. Arginine Dihydrolase and Catalase can be harnessed from the isolates and marketed as molecular biological resource for research. Antibiotic susceptibility tests (Table-4) indicated midgut microbiota of *Culex* mosquitoes obtained from Diamond Harbour areas are found to be moderately resistant to the antibiotics such as polymyxin-B and Amikacin. and they are more the less sensitive against all other antibiotics used. These results reveal that the availability of mid gut microbiota of mosquitoes may indicate the presence of these bacteria within the host (Human being/ cattle) of mosquitoes. Mosquitoes are known to illicit a specific immune response against parasite, Gram positive and Gram negative bacteria. Some of the immune responsive genes are expressed in response to both protozoa and bacteria (Bauer et al, 1966). Several lines of data (Kasai et al, 1986; Kumar et al, 2013;) have indicated that midgut microbiota of *Culex* mosquitoes stimulate basal immune activity which in turn inhibit the growth of parasites viz *Wuchereria*, *Plasmodium* etc. It can suggest that this microbiota may also illicit basal immunity of the host (human/ cattle). *Culex* mosquitoes 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. The mid gut bacterial diversity along with the biochemical characteristic is closely associated with the complex potential interaction between the symbiotic microbes and host. Current technologies are

not sufficient to pinpoint all the fluxes of matter and energy between microorganisms and their hosts. However, a little investigation has been done on the beneficial functions provided by bacteria, especially those living intracellularly as endosymbionts

(Moffatt et al 2010). It can be concluded that the study on gut microbiota along with their different biochemical characteristics may open new windows for better understanding.

Table.1 Morphotypes of isolated colony

Sample	Morphotype	Morphological Characters of colony						No. of isolates selected
		shape	Size	colour	Visibility and texture	elevation	type	
C1	M1	circular	2-3mm in diameter	brown	Opaque, matte	plain	Diffuse, margin entire	5
C2	M1	circular,	2mm in diameter,	yellow	Opaque, matte	convex	Diffuse, margin entire	5
C3	M1	circular	2-3mm in diameter	whitish	Opaque, matte	plain	Diffuse, margin entire	4
C4	M1	umbonate	2-3mm in diameter	creamish	Opaque, glistening	plain	Diffuse, margin entire	5
C5	M1	umbonate	2-3mm in diameter	whitish	Opaque, glistening	plain	Diffuse, margin entire	3
C5	M2	circular	2-3mm in diameter	whitish	Opaque glistening	plain	Diffuse, margin entire	4

Table.2 Gram character, morphology and arrangement of cells in each morphotype

Isolates from morphotype	Gram Character	Morphology and arrangement
C1M1	Gram Positive	Coccus shaped, mostly in chains.
C2M1	Gram Positive	Coccus shaped, mostly in pair.
C3M1	Gram Positive	Coccus shaped, mostly in pair.
C4M1	Gram Positive	Coccus shaped, clumped, held together.
C5M1	Gram Positive	Coccus shaped, singly arranged.
C5M2	Gram Positive	Coccus shaped, mostly in pair.

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 Bassay	Endo agar assay	Amylase Production assay	Protease Production assay
C1M1	-VE	+VE	-VE	-VE	-VE	+VE	-VE	+VE	-VE
C2M1	-VE	-VE	-VE	-VE	-VE	-VE	-VE	+VE	-VE
C3M1	+VE	-VE	-VE	-VE	-VE	+VE	-VE	+VE	+VE
C4M1	-VE	-VE	+VE	+VE	-VE	+VE	-VE	+VE	-VE
C5M1	-VE	-VE	-VE	-VE	+VE	+VE	-VE	+VE	-VE
C5M2	-VE	-VE	-VE	-VE	+VE	+VE	-VE	+VE	-VE

Table.4 Assessment of antibiotic sensitivity assay

Sample number	Antibiotic sensitivity patterns (Resistant/Intermediate/Sensitive) as per CLSI standards								
	Ticarcillin	Gentamicin	Imipenem	Ciprofloxacin	Polymyxin-B	Colistin	Netillin	Tetracyclin	Amikacin
C1M1	Sensitive	Sensitive	Sensitive	Sensitive	Sensitive	Sensitive	Sensitive	sensitive	Sensitive
C2M1	Sensitive	Sensitive	Sensitive	Sensitive	Resistant	Sensitive	Sensitive	Sensitive	Sensitive
C3M1	Sensitive	Sensitive	Sensitive	Sensitive	Sensitive	Sensitive	Sensitive	Sensitive	Sensitive
C4M1	Sensitive	Sensitive	Sensitive	Sensitive	Sensitive	Sensitive	Sensitive	Sensitive	Intermediate to Sensitive
C5M1	Sensitive	Sensitive	Sensitive	Sensitive	Sensitive	Sensitive	Sensitive	Sensitive	Sensitive
C5M2	Sensitive	Sensitive	Sensitive	Sensitive	Sensitive	Sensitive	Sensitive	Sensitive	Sensitive

Figure.1 Map of South 24 Parganas showing the collection site-Diamond Harbour (www.mapsofindia.com)



Fig.2 Morphology of the six axenic isolates, from top left to bottom right representing C1M1,C2M1,C3M1,C4M1,C5M1,C5M2 respectively

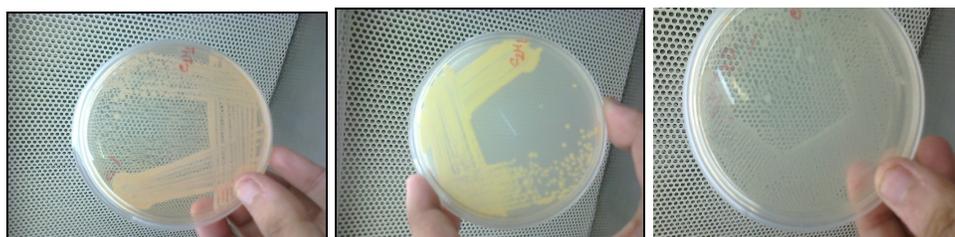
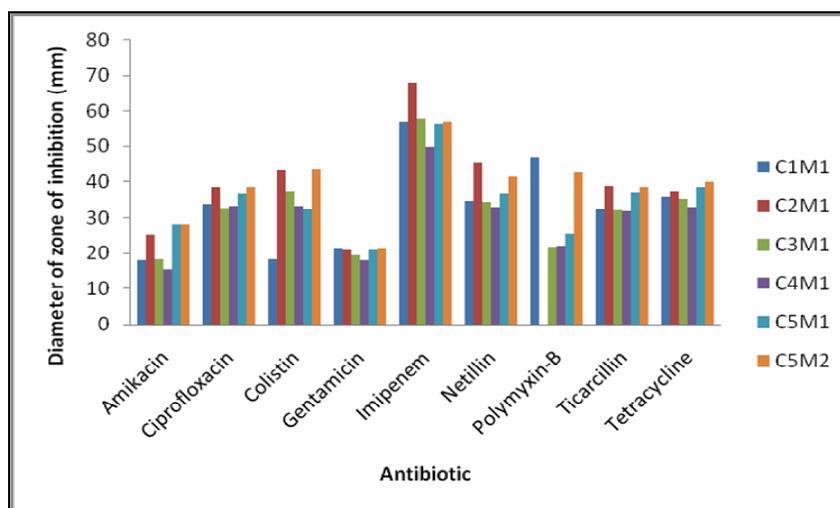




Figure.3 Antibiogram of mean zone inhibition of gut bacterial isolates from each morphotype of five sample against nine antibiotics



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