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Studies on mid gut microbiota of wild caught *Culex* (*Culex quinquefasciatus*) mosquitoes from Barasat (North 24 Parganas) of West Bengal

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ABSTRACT
Mosquitoes are haematophagous insects that serve as obligate intermediate host for numerous diseases like Filaria, Malaria, Dengue, etc. Mosquitoes can be considered as a holobiont units in which host (mosquitoes) and its gut microbiota are involved in a complex reciprocal interaction. The naturally acquired microbiota can modulate mosquitos' vectorial capacity by inhibiting the development of pathogens. But, enough care has not been taken in West Bengal to investigate on the midgut microbiota of *Culex* mosquitoes. Therefore, a preliminary attempt has been undertaken to study the morphology, growth pattern and antibiotic susceptibility of midgut microbiota of *Culex* (*Culex quinquefasciatus*) mosquitoes collected from Barasat areas (North 24 Parganas) of West Bengal.

Keywords: Antibiotic susceptibility, Bacterial growths, *Culex*, Gut-microbiota.

1. Introduction
Mosquitoes, the known group of haematophagous insect, serve as the obligate intermediate host for numerous diseases like Filaria, Malaria, Dengue etc. that cause human mortality and morbidity worldwide [16]. *Culex quinquefasciatus* is the principal vector of filariasis and Japanese Encephalitis (JE) in India. 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 [5]. All insect species are known to harbor a rich and complex community of microorganisms in their guts and other body regions. The gut microbiota display different types of interactions ranging from pathogenesis to obligate mutualism [7]. Diversity of feeding habit along with the structural variations of gut promotes and establishes different phylotypes of gut microbiota [5]. Various lines of data [3, 21, 22] 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 are thought to be an effective strategy for controlling the spread of pathogens that use insects as hosts [2, 8, 13]. *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 with the *Culex* mosquitoes in various regions of West Bengal. In view of these reasons, a preliminary investigation was undertaken 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*) *m*osquitoes in Barasat (North 24 Parganas) areas of West Bengal.

2. Materials and methods:
2.1 Collection of mosquitoes
*Culex quinquefasciatus m*osquitoes were collected early morning during the year 2013 (March) to 2014 (February) from Barasat areas (Fig-1) of West Bengal (Latitude: 22.5667° N, Longitude: 88.3667°E) using an aspirator. The collected samples were transported to the laboratory in a sterile glass bottle with perforated cap. Only live mosquitoes were selected for microbiological analysis.

Fig 1: Map of North 24 Parganas showing the collection site-Barasat

2.2 Isolation of bacteria

In each experiment, two mosquito samples were initially killed with chloroform and then surface sterilized using 70% ethyl alcohol. The guts of the samples were removed using sterile forceps and then mixed thoroughly with 500 µl of sterile physiological saline [0.7% (wv^{-1}) aqueous solution of sodium chloride, pH 7.2] in a sterile watch glass inside a laminar air-flow bench. One loopfull of midgut suspension was then streaked on the surface of a sterile and dried nutrient agar (HiMedia M1269) plate. Three replica plates were prepared from each sample. The plates were then incubated at 30 °C for 24 hours for appearance of isolated colonies. The isolated colonies were selected based on their morphology. The selected morphotypes were purified twice on nutrient agar plates and two to five cultures of each morphotype were stored in nutrient agar slants at 4 °C for their future characterization.

2.3 Gram staining of microorganisms

Gram staining of each culture was carried out following the procedure of Harrigan and MacCance, 1976 [10].

2.4 Study of bacterial growth

Single culture of each morphotype was selected for generation of growth curve. Before inoculation each culture was grown for 6hours at 30 °C in nutrient broth (HiMedia M002). Briefly 250 µl of each of the cultures was inoculated to 25ml of sterile nutrient broth kept in 100 ml Erlenmeyer flask. The flasks were incubated at 30 °C with 200 rpm in a shaking incubator and optical density measured at 600nm at 1.5hours interval for a duration of 10.5hours using Jasco UV-visible spectrophotometer (model V600) using a quartz cuvette (Kozima) [17]. Uninoculated nutrient broth served as control. The obtained results were plotted to generate growth curve.

2.5 Antibiotic sensitivity assay

A total of seven strains of bacteria isolated from two samples of *Culex* mosquito were tested for their antibiotic resistance pattern against nine antibiotics. Each experiment was performed thrice for perfection of the result. Antibiotic impregnated paper discs from HiMedia used for the study include amikacin (30 µg $disc^{-1}$), ciprofloxacin (5 µg $disc^{-1}$), colistin (10 µg $disc^{-1}$), gentamicin (10 µg $disc^{-1}$), imipenem (10 µg $disc^{-1}$), netillin (30 µg $disc^{-1}$), polymyxin-B (300 µg $disc^{-1}$), tetracycline (30 µg $disc^{-1}$) and ticarcillin (75 µg $disc^{-1}$). Each bacterial culture was previously grown on sterile nutrient agar plate at 30 °C for 24 hours to generate isolated colonies. Colonies (3-5) of each culture were grown in nutrient broth for 6 hours at 30 °C. After work, lawn on Muellar-Hinton Agar (HiMedia M173) plates using cotton swab was prepared. After drying for fifteen minutes, the selected antibiotic discs were placed aseptically using sterile forceps kept at a distance of 4 cm between their centres. Complete inhibition zone around each disc was measured after 18 hours of incubation at 30 °C [1].

3. Observation/Result:

3.1 Bacterial isolates

Three morphotypes of bacterial colony were selected from one sample of mosquito while four from the other sample. The characters of each morphotype and the number of isolates studied from each morphotype are presented in Table 1.

Table 1: Morphotypes of isolated colony

Sample	Morphotype	Morphological characters	Number of selected colonies
B1	M1	1-2mm in diameter, whitish in colour, transluscent, glistening, surface slightly convex, margin smooth	5
	M2	1-2mm in diameter, creamish in colour, opaque, glistening, surface slightly convex, margin smooth	3
	M3	3-4mm in diameter, whitish in colour, transparent, more glistening, surface flat, margin smooth	2
B2	M1	1-2mm in size, whitish in colour, opaque, slightly rough texture, surface flat, margin irregular,	5
	M2	1-2mm in size, whitish in colour, transluscent, non-glistening, surface flat, margin smooth	3
	M3	3-4mm in size, whitish in colour, opaque, slightly rough texture, surface flat, margin irregular, spreading from the streak line.	2
	M4	1-2mm in size, yellowish in colour, transluscent, glistening, surface convex, margin smooth	4

3.2 Gram characteristics of microorganism

Of the three morphotypes tested from sample B1, organisms from two morphotypes showed positive Gram reaction while one showed negative Gram reaction. From sample B2 out of four morphotypes, organisms from one were Gram positive in nature while three were Gram negative. Gram character, morphology and arrangement of cell in each morphotype are shown in Table 2.

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

Isolates from morphotype	Gram character	Morphology and arrangement
B1M1	Gram positive	Rod shaped, mostly single, some in pair
B1M2	Gram positive	Rod shaped, mostly single
B1M3	Gram negative	Short rod in shape, mostly single
B2M1	Gram positive	Large rod, mostly single and some in pair
B2M2	Gram negative	Small rod, singly arranged
B2M3	Gram negative	Small rod, singly arranged
B2M4	Gram negative	Small rod, mostly single, some in pair

3.3 Growth of bacterial isolates

Growth curve of bacterial isolates from each of the three morphotypes of sample B1 and bacterial isolates from each of the four morphotypes of sample B2 are shown in Table 3 and the graphical representation in Figure 2 and 3.

Table 3: Measurement of growth rate of isolates

Time (hours)	00	1.5	3	4.5	6	7.5	9	10.5
Sample no.	Optical density at 600 nm							
B1M1	0.0108	0.2778	0.7258	1.4023	1.7232	1.9084	1.9578	1.9575
B1M2	0.0097	0.2892	0.7604	1.2792	1.5105	1.7180	1.7982	1.8211
B1M3	0.0004	0.1630	0.4680	1.1850	1.3254	1.6095	1.7341	1.7449
B2M1	0.0623	0.1925	0.5305	1.3145	1.5420	1.7326	1.7782	1.7795
B2M2	0.0098	0.1516	0.2421	0.4815	0.7844	1.3918	1.6532	1.6629
B2M3	0.0042	0.1429	0.4920	1.2243	1.4528	1.7221	1.8254	1.8266
B2M4	0.0112	0.1021	0.1265	0.3590.	0.6320	1.1824	1.3345	1.3629

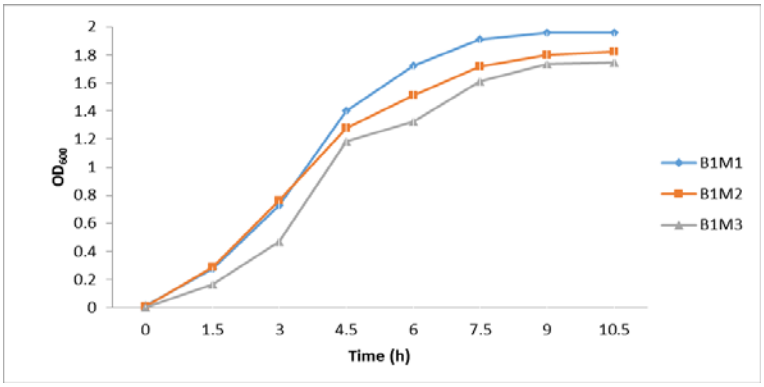

Fig 2: Growth curve of gut bacterial isolates from each of the three morphotypes of sample B1

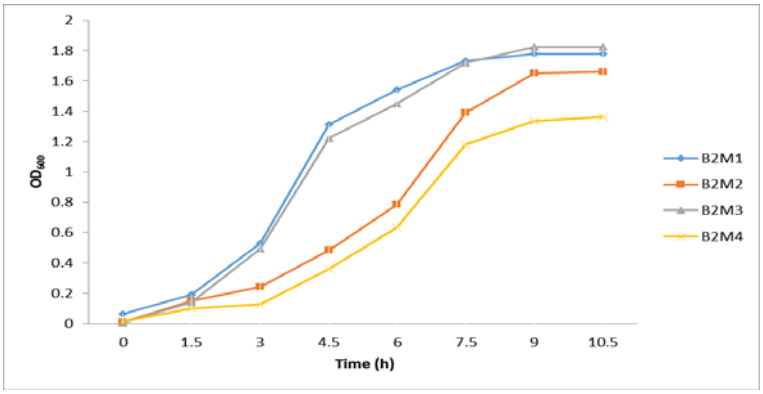

Fig 3: Growth curve of gut bacterial isolates from each of the four morphotypes of sample B

3.4 Antibiotic susceptibility

Mean zone inhibition of bacterial isolates from each of the three morphotype of sample B1 against nine antibiotics are presented in Table 4 and Figure 5.

Mean zone inhibition of bacterial isolates from each of the four morphotype of sample B2 against nine antibiotics are presented in Table 4 and Figure 6.

Table 4: Assessment of antibiotic sensitivity assay

Sample number	Ticarcillin	Gentamicin	Imipenem	Ciprofloxacin	Polymyxin-B	Colistin	Netillin	Tetracyclin	Amikacin
B1M1	i) 9 mm ii) 9mm iii)8mm	i) 18 mm ii) 19 mm iii) 18mm	i) 19 mm ii) 19 mm iii) 18mm	i) 31 mm ii) 32 mm iii) 32 mm	i) 12 mm ii) 12 mm iii) 13mm	i) 10mm ii)10mm iii)10mm	i) 20mm ii) 21mm iii 20mm	i)15mm ii)16mm iii) 15m	i) 19 mm ii)17 mm iii)19mm
Mean zone of inhibition (in mm)	8.67	18.33	18.67	31.67	12.33	10	20.3	15.33	18.33
Resistant / sensitive type	Resistant	Sensitive	Sensitive	Sensitive	Sensitive	Resistant	Sensitive	Intermediate to sensitive	Sensitive
B1M2	i) 9 mm ii) 9mm iii)10mm	i) 17 mm ii) 18 mm iii) 16mm	i) 16 mm ii) 17 mm iii) 16mm	i) 24 mm ii) 24 mm iii) 26mm	i) 12 mm ii) 13 mm iii) 13 mm	i) 9 mm ii) 9 mm iii) 9 mm	i) 19 mm ii) 19 mm iii) 18 mm	i) 19 mm ii) 19 mm iii) 19 mm	i) 19 mm ii) 20 mm iii) 19 mm
Mean zone of inhibition (in mm)	9.33	17	16.33	24.67	12.67	9	18.67	19	19.33
Resistant / sensitive type	Resistant	Sensitive	Sensitive	Sensitive	Sensitive	Resistant	Sensitive	Sensitive	Sensitive
B1M3	i) 14 mm ii) 13 mm iii) 13 mm	i) 22 mm ii) 24 mm iii) 23 mm	i) 29 mm ii) 28 mm iii) 29 mm	i) 31mm ii) 34 mm iii) 34 mm	i) 14mm ii)13mm iii)13 mm	i) 14mm ii)13mm iii)13 mm	i) 23 mm ii) 24 mm iii) 23 mm	i) 20 mm ii) 21 mm iii) 21 mm	i) 23 mm ii) 24 mm iii) 21 mm
Mean zone of inhibition (in mm)	13.33	23	28.67	33	13.33	13.33	23.33	20.67	22.67
Resistant / sensitive type	Resistant	Sensitive	Sensitive	Sensitive	Sensitive	Sensitive	Sensitive	Sensitive	Sensitive
B2M1	i) 5 mm	i) 25 mm	i) 38 mm	i) 31 mm	i) 14 mm	i) 10 mm	i) 25 mm	i) 22 mm	i) 27 mm

	ii) 6 mm iii) 5 mm	ii) 25 mm iii) 24 mm	ii) 39 mm iii) 38 mm	ii) 32 mm iii) 32 mm	ii)13 mm iii)14 mm	ii) 10 mm iii) 10 mm	ii) 25 mm iii) 24 mm	ii) 22 mm iii) 21 mm	ii) 27 mm iii) 26 mm
Mean zone of inhibition (in mm)	5.33	24.67	38.33	31.67	13.67	10	24.67	21.67	26.67
Resistant / sensitive type	Resistant	Sensitive	Sensitive	Sensitive	Sensitive	Resistant	Sensitive	Sensitive	Sensitive
B2M2	i) 29 mm ii) 28 mm iii) 29 mm	i) 25 mm ii) 24 mm iii) 25 mm	i) 40 mm ii) 41 mm iii) 40 mm	i) 34 mm ii) 35 mm iii) 34 mm	i) 13 mm ii) 14 mm iii) 14 mm	i) 6 mm ii) 7 mm iii) 6 mm	i) 27 mm ii) 27 mm iii) 26 mm	i) 25 mm ii) 27 mm iii) 27 mm	i) 25 mm ii) 24 mm iii) 25 mm
Mean zone of inhibition (in mm)	28.67	24.67	40.33	34.33	13.67	6.33	26.67	26.33	24.67
Resistant / sensitive type	Sensitive	Sensitive	Sensitive	Sensitive	Sensitive	Resistant	Sensitive	Sensitive	Sensitive
B2M3	i) 00 mm ii) 00 mm iii) 00 mm	i) 25 mm ii) 24 mm iii) 24 mm	i) 36mm ii) 35 mm iii) 36 mm	i) 27 mm ii) 29 mm iii) 28 mm	i)14 mm ii)14 mm iii)13 mm	i) 4 mm ii) 3 mm iii) 4 mm	i) 24 mm ii) 25 mm iii) 24 mm	i) 23 mm ii) 23 mm iii) 22 mm	i) 26 mm ii) 27 mm iii) 27 mm
Mean zone of inhibition (in mm)	00	24.33	35.67	28	13.67	3.67	24.33	22.67	26.67
Resistant / sensitive type	Resistant	Sensitive	Sensitive	Sensitive	Sensitive	Resistant	Sensitive	Sensitive	Sensitive
B2M4	i) 22 mm ii) 23 mm iii) 24 mm	i) 27 mm ii) 28 mm iii) 27 mm	i) 26 mm ii) 24 mm iii) 25 mm	i) 45 mm ii) 46 mm iii) 45 mm	i) 17 mm ii) 17 mm iii) 16 mm	i) 15 mm ii) 16 mm iii) 14 mm	i) 27 mm ii) 28 mm iii) 26 mm	i) 27 mm ii) 27 mm iii) 26 mm	i) 25 mm ii) 24 mm iii) 25 mm
Mean zone of inhibition (in mm)	23	27.33	25	45.33	16.67	15	27	26.67	24.67
Resistant / sensitive type	Sensitive	Sensitive	Sensitive	Sensitive	Sensitive	Sensitive	Sensitive	Sensitive	Sensitive

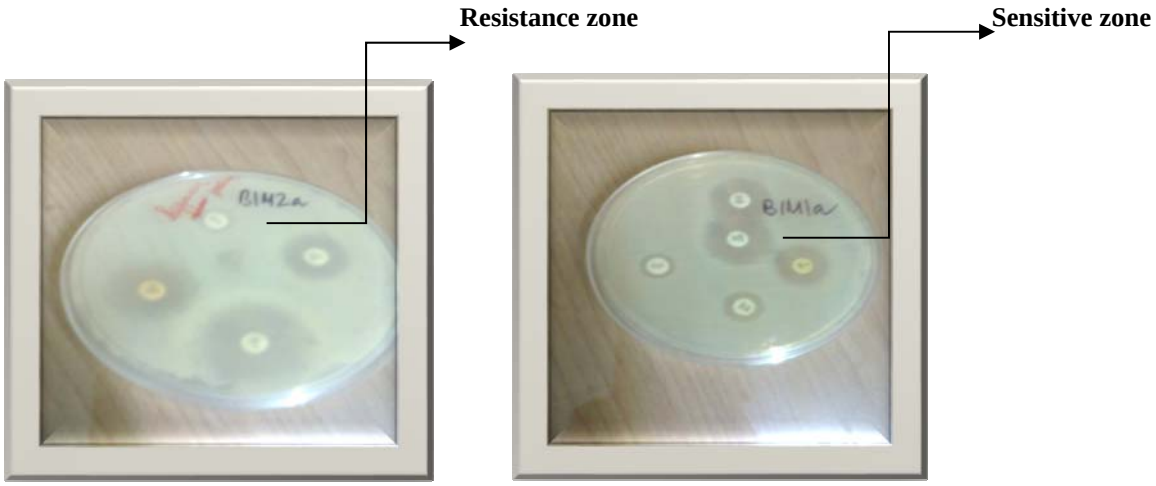

Fig 4: Showing antibiotic resistance and sensitive zone

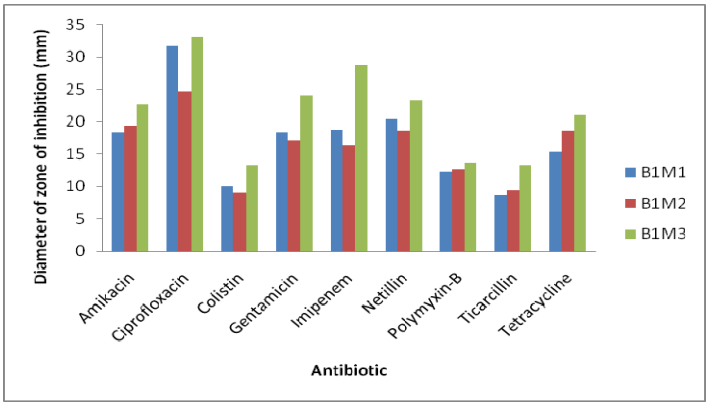

Fig 5: Bar diagram showing mean zone inhibition of three gut bacterial isolates from each Morphotype of sample B1 against nine antibiotics.

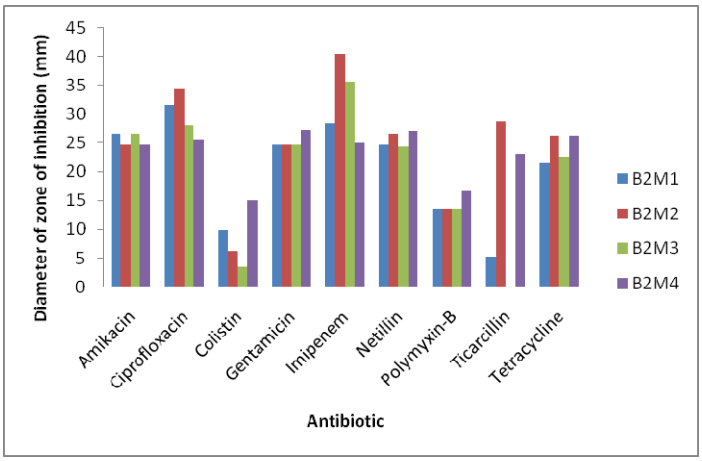

Fig 6: Bar diagram showing mean zone inhibition of four gut bacterial isolates from each Morphotype of sample B2 against nine antibiotics.

4. Discussion:

Results of the present study provided comprehensive information about colony character, Gram staining properties, growth pattern and antibiotic sensitivity of mid gut microbiota of *Culex* mosquitoes collected from Barasat areas of West Bengal (Fig -1). Data (Table-1) revealed that the gut-bacterial colonies are more or less convex having smooth margin. Data (Table-2) also indicated that of seven bacterial isolates, four isolates were Gram negative and three Gram positive in nature and all of them were rod shaped with long or short in length. Some of the morphotypes of gut bacterial isolates showed slow growth rate and rest of them displayed comparatively rapid growth rate (Fig: 2 and 3). It has been reported that the rapid growth of mid gut microbiota may help in blood digestion within the gut of mosquitoes [18]. Biochemical test regarding antibiotic assay of morphotypes against different antibiotics (Fig:4) indicated that midgut microbiota of *Culex* mosquitoes obtained from Barasat areas of West Bengal were found to be highly resistant to antibiotics *viz*., colistin and ticarcillin and mostly sensitive to antibiotic gentamicin, imipenem, ciprofloxacin, polymyxin-B , netillin , tetracyclin and amikacin (Fig:5 and Fig:6). It has been reported [11] that those gut bacteria were resistant to ticarcillin and may express beta-lactamases which cleave beta lactam ring of ticarcillin. Another antibiotic was colistin, against which most of the gut bacterial isolates showed resistance. Earlier investigation revealed that colistin was polycationic antibiotic having both hydrophilic and lipophilic moieties. These poly-cationic regions interact with the bacterial outer membrane by displacing bacterial counter ions in the lipopolysaccharide. The resistance may be due to the modification or loss of the polysaccharide portion of LPS, due to which the drug may not easily displace the ions, favouring survival of the bacteria in presence of drugs [14]. Mosquitoes are known to elicit a specific immune response against parasites, Gram positive and Gram negative bacteria. Some of the immune responsive genes are expressed in response to both protozoa and bacteria [18]. Several studies [9, 12, 20] have indicated that midgut microbiota of mosquitoes stimulate basal immune activity which in turn inhibit the growth of parasites *viz*., *Wuchereria* and *Plasmodium*.

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 [4].The mid gut bacterial diversity along with the biochemical characteristics were closely associated with the complex potential interaction between the symbiotic microbes and host[12]. Modern technologies are not sufficient to pinpoint all the fluxes of matter and energy between microorganisms and their hosts. However, little research work has been forwarded towards the beneficial functions served by the intracellularly living endosymbiont bacteria. It can be concluded that the present study on gut microbiota along with their antibiotic resistance and different biochemical characteristics may open new windows for better understanding of *Culex*-midgut microbiota interaction.

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