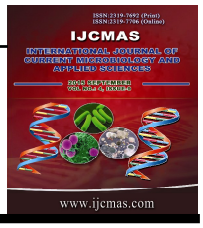
International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 4 Number 9 (2015) pp. 711-725**
http://www.ijcmas.com



Original Research Article

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

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Keywords

Anopheles, Gut microbiota, Biochemical characterization, Antibiotic susceptibity

A B S T R A C T

Mosquitoes are haematophagous vector and carries parasites and pathogens of numerous diseases like Filaria, 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 *Anopheles* mosquitoes. Therefore a preliminary attempt has been undertaken to study the morphology, biochemical characterization and antibiotic susceptibility of midgut microbiota of *Anopheles* (*An vagus& Ansubpictus)* mosquitoes collected from Diamond Harbour (South 24 Parganas) areas of west Bengal.

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 (Moffatt *et al.,* 2010). 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 (Das, 1997). 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 (Dillon and Dillon, 2004). Diversity of feeding habit along with the structural variations of gut promotes and establishes different phylotypes of gut microbiota (Das, 1997). Various lines of data (Chernysh *et al.,* 2002; Pal *et al.,* 2015; Wilkinson, 2001; Zhang and Brune, 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 (Beard *et al.*, 2002; Dillon *et al.*, 2005; Lehane *et al.*, 1997; Pal *et al.*, 2015). Manipulating these microbial symbionts are thought to be an effective strategy for controlling the spread of pathogens that use insects as hosts (Pumpuni *et al.*, 1996). *Anopheles*, an anthropophilic vector, is not only responsible for the transmission of Malaria 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 *Anopheles* mosquitoes in various regions of West Bengal. In view of these reasons, a preliminary investigation has undertaken during the year 2014-2015 to find out morphological characteristics such as Gram staining, pattern of microbial growth and antibiotic susceptibility assay, Biochemical pathways of mid gut bacterial isolates of *Anopheles* (*An.subpictus& An.vagus*) *m*osquitoes in Diamond Harbour (South 24 Parganas) areas of West Bengal.

Materials and Methods

Collection of mosquitoes

Anopheles mosquitoes were collected between 5.30 to 6.30 am 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 (22.2°N, 88.2°E) (Fig. 1) 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.

Isolation of bacteria and grams staining

Mosquito samples (*An.vagus* & *An.subpictus*) were killed with chloroform, surface sterilized with 70% ethanol (v/v), their guts removed and mixed thoroughly with 500 µl of sterile physiological saline [0.7% (w/v), pH 7.2]. One loopfull of midgut suspension was then streaked on the surface of a sterile nutrient agar (HiMedia) plates, and incubated at 30 °C for 24 hours for appearance of isolated colony morphotypes. The experiment was done in triplicate and subsequent axenic cultures stored at 4°C for further assays.

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

DNase production detection assay

Overnight (18-20 hrs.) Nutrient broth culture suspensions of the isolates were inoculated on 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^{o}C 7.2 ± 0.2) and incubated at 35^{o}C ± 2^{o}C for 18 to 48 hours.

Citrate production assay

Overnight (18–20 hrs.) Nutrient broth culture suspensions of the isolates were inoculated on sterile Simmons citrate agar medium (Magnesium sulphate 0.200 gms/ltr, Ammonium dihydrogen phosphate 1.000 gms/ltr, Dipotassium phosphate 1.000 gms/ltr, Sodium citrate 2.000 gms/ltr, Sodium chloride 5.000 gms/ltr, Bromothymol blue 0.080 gms/ltr, Agar 15.000 gms/ltr, Final pH (at 25°C) 6.8±0.2) and incubated at 35^{o}C ± 2^{o}C for 18 to 48 hours.

Phosphate-solubilizing assay

Overnight (18-20 hrs.) nutrient broth culture

suspensions of the isolates were inoculated on Sterile Pikovskaya agar medium (Yeast extract 0.500 gms/ltr, Dextrose 10.000 gms/ltr, Calcium phosphate 5.000gms/ltr, Ammonium sulphate 0.500 gms/ltr, Potassium chloride 0.200 gms/ltr, Magnesium sulphate 0.100 gms/ltr, Manganese sulphate 0.0001gms/ltr, Ferrous sulphate 0.0001 gms/ltr, Agar 15.000 gms/ltr) and incubated at 35^{o}C ± 2^{o}C for 18 to 48 hours.

Methyl red test

Glucose Phosphate broth (Buffered peptone 7.000 gms/ltr, Dextrose 5.000 gms/ltr, Dipotassium phosphate 5.000 gms/ltr, final pH (at 25°C) 6.9±0.2 culture suspensions of the isolates were prepared in culture tube and incubated at 35^{o}C ± 2^{o}C for 18 to 48 hours. followed by a few drops of Methyl Red with an incubation of 5–10 mins.

Indole production assay

Overnight (18-20 hrs.) Tryptone Broth [Casein enzymichydrolysate 10.000 gms/ltr, Sodium chloride 5.000 gms/ltr, Final pH (at 25°C) 7.5±0.2] culture suspensions of the isolates were prepared in culture tube and incubated at 35^{o}C ± 2^{o}C for 18 to 48 hours, followed by a few drops of Kovac'sindole reagent with an incubation of 5-10 mins.

Voges Proskauer Test

Glucose Phosphate broth [Buffered peptone 7.000 gms/ltr, Dextrose 5.000 gms/ltr, Dipotassium phosphate 5.000 gms/ltr, Final pH (at 25°C) 6.9±0.2] culture suspensions of the isolates were prepared in culture tube and incubated at 35^{o}C ± 2^{o}C for 18 to 48 hours followed by 150 µl of Barritt's-A & 60µl of Barritt's-B with an incubation of 5-10 mins.

Antibiotic sensitivity assay

The bacterial isolates were tested for their antibiotic resistance pattern against eight commercially available antibiotic impregnated paper discs (HiMedia) used for the study viz., ciprofloxacin (5µg), colistin (10µg), gentamicin (10µg), imipenem (10µg), netillin (30µg), polymyxin-B (300µg), tetracycline (30µg) and ticarcillin (75µg). Isolated bacterial colonies of each isolates were grown in nutrient broth for 6 hours 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. Each experiment was performed in triplicate.

Results and Discussion

Bacterial isolates

Total 9 morphotypes of bacteria selected (2 morphotypes from one *An. vagus*, 3 morphotypes from another *An. vagus* and 4 morphotypes from *An. subpictus).* The characters of each morphotype and the number of isolates studied from each morphotype are presented in table 1.

Gram staining of microorganisms

Gram character, morphology and arrangements of cells in each morphotypes are shown in table 2.

DNase production detection assay

DNase production assay results of the isolates are given below in table 3.

Citrate production assay

The result of citrate production assay of the isolates is presented in table 4 & pictures are given below.

Phosphate-solubilizing assay

Phosphate solubilizing assay show negative result in all isolates, it is shown in table 5.

MR test

Results of Methyl Red test are present in table 6 & pictures are given below.

VP test

VP test was done simultaneously with Methyl Red test by the Barritt's- A & Barritt's- B reagents. The results are given below in table 8.

Antibiotic sensitivity assay

Results of antibiotic sensitivity assay are shown in table 9.1, sensitive area measurements are shown in table 9.2 & a graph of sensitivity assay is shown below in figure 8.

It has been reported that microbe free aseptic mosquitoes displayed increase susceptibility to *Plasmodium* infection while co-feeding mosquitoes with bacteria and *Plasmodium falciparum* gametocytes resulted in lower than normal infection (Dong *et al.,* 2009). Our studies have been extended to know the biochemical characteristics of *Anopheles* mosquitoes. Results of the present study (Table 1 and 2) provide comprehensive information about colony character, Gram staining properties and antibiotic susceptibility status of mid gut microbiota of *Anopheles* (*An. vagus* & *An.subpictus*) mosquitoes from the Diamond harbour region of South 24 Parganas of West Bengal. Present observations reveal that most of the gut bacterial isolates are gram positive in nature and their colonies are more or less convex with smooth margin. Several biochemical tests have been performed to know the biochemical nature of mid gut microbiota of *Anopheles* mosquitoes. The result of biochemical tests (Table 3, 4, 6 and 8) regarding DNase production assay, citrate production assay, Methyl Red test and Voges-Proskauer test provide more or less positive biochemical reaction. Our study reveal that the gut microbiota have the ability to metabolize Citrate as sole carbon source, production of acids from metabolism of lactose via mixed acid fermentation and production of acid via butanediol fermentation respectively. On the contrary gut bacterial isolates are failed to show any positive reaction (Table 5 & 7) in phosphate solubilizing assay and inindole production assay respectively.

The present study investigation has been extended to know the antibiotic sensitivity status of mid gut bacterial isolates of *Anopheles*. The data (Table 9.1) reveal that the gut bacterial isolates are mostly sensitive to antibiotics colistin, gentamicin, imipenem, ciprofloxacin, polymyxin-B, netillin, tetracycline & amikain and resistant to antibiotic ticarcillin. It has been reported that those gut bacteria are resistant to ticarcillin and may express beta-lactamases which cleave beta lactam ring of ticarcillin (Kasai *et al.,* 1986; Pal *et al.,* 2015). 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 (Pidiyar *et al.,* 2004). Mosquitoes are known to elicit a

specific immune responsive against parasites and bacteria (Gram positive and Gram negative). Some of the immune responsive genes are expressed in response to both protozoa and bacteria (Dong *et al.*, 2009). Several studies (Clements, 1999; Kumar *et al.*, 2013; Pumpuni *et al.*, 1996; Pal *et al.*, 2015) have indicated that midgut microbiota of mosquitoes stimulate basal immune activity which in turn inhibit the growth of parasites viz. *Plasmodium vivax* & *Plasmodium falciparum*.

Table.1 Morphotypes of isolated colony

Zoonose	Name of morphotypes	Characters
An. vagus	DA_2M_1	1-2mm in diameter, whitish in colour, transluscent, glistening, surface slightly convex, margin smooth.
	DA_2M_2	1-2mm in diameter, creamish in colour, opaque, glisting, serface slightly convex, margin smooth.
An. vagus	DA_3M_1	3-4mm in diameter, whitish in colour, transparent, more glisting, surface flat, margin smooth.
	DA_3M_2	1-2mm in size, whitish in colour, opaque, slightly rough texture, surface flat, margin irregular
	DA_3M_3	1-2mm in size, whitish in colour, transluscent, non-glistening, surface flat, margin smooth
An. subpictus	D_2AM_1	5-6mm in diameter, whitish in colour, opaque, smooth texture, surface flat, margin smooth
	D_2AM_2	1-2mm in diameter, creamish in colour, opaque, glisting, serface slightly convex, margin smooth.
	D_2AM_3	6-7mm in diameter, yellowish in colour, opaque, smooth texture, surface convex, margin smooth.
	D_2AM_4	3-4mm in diameter, slightly whitish colour, transiuscent, glisting, surface slightly convex, margin irregular.

Table.2 Morphological characteristics of organisms

GRAM CHARACTERISTICS OF THE MICROORGANISMS		
Isolates from morphotype	**Gram character**	**Morphology & arrangement**
DA_2M_1	Gram positive	Coccus shaped, mostly in chains.
DA_2M_2	Gram positive	Coccus shaped, mostly in pair.
DA_3M_1	Gram negative	Short rod in shape, mostly single
DA_3M_2	Gram negative	Small rod, mostly single, some in pair
DA_3M_3	Gram positive	Coccus shaped, clumped, held together.
D_2AM_1	Gram positive	Coccus shaped, singly arranged.
D_2AM_2	Gram negative	Short rod in shape, mostly single
D_2AM_3	Gram positive	Coccus shaped, mostly in pair, some single.
D_2AM_4	Gram positive	Coccus shaped, clumped, held together.

Table.3 DNase production assay

DNase PRODUCTION ASSAY	
SAMPLE NO	DNAse PRODUCTION
DA_2M_1	Negative
DA_2M_2	Positive
DA_3M_1	Negative
DA_3M_2	Negative
DA_3M_3	Positive
D_2AM_1	Negative
D_2AM_2	Negative
D_2AM_3	Positive
D_2AM_4	Positive

Table.4 Citrate production assay

CITRATE PRODUCTION ASSAY	
SAMPLE NO	CITRATE PRODUCTION
DA_2M_1	NEGATIVE
DA_2M_2	NEGATIVE
DA_3M_1	NEGATIVE
DA_3M_2	NEGATIVE
DA_3M_3	NEGATIVE
D_2AM_1	POSITIVE
D_2AM_2	NEGATIVE
D_2AM_3	NEGATIVE
D_2AM_4	NEGATIVE

Table.5 Phosphate solubilizing assay

PHOSPHATE SOLUBILIZING ASSAY	
SAMPLE NO	CITRATE PRODUCTION
DA_2M_1	NEGATIVE
DA_2M_2	NEGATIVE
DA_3M_1	NEGATIVE
DA_3M_2	NEGATIVE
DA_3M_3	NEGATIVE
D_2AM_1	NEGATIVE
D_2AM_2	NEGATIVE
D_2AM_3	NEGATIVE
D_2AM_4	NEGATIVE

Table.6 Methyl Red TEST

Methyl Red TEST	
SAMPLE NO	RESULTS
DA_2M_1	NEGATIVE
DA_2M_2	NEGATIVE
DA_3M_1	POSITIVE
DA_3M_2	POSITIVE
DA_3M_3	POSITIVE
D_2AM_1	NEGATIVE
D_2AM_2	POSITIVE
D_2AM_3	NEGATIVE
D_2AM_4	POSITIVE

Table.7 Indole production assay

INDOLE PRODUCTION ASSAY	
SAMPLE NO	RESULTS
DA_2M_1	NEGATIVE
DA_2M_2	NEGATIVE
DA_3M_1	NEGATIVE
DA_3M_2	NEGATIVE
DA_3M_3	NEGATIVE
D_2AM_1	NEGATIVE
D_2AM_2	NEGATIVE
D_2AM_3	NEGATIVE
D_2AM_4	NEGATIVE

Table.8 VP test

VP TEST	
SAMPLE NO	RESULTS
DA_2M_1	NEGATIVE
DA_2M_2	POSITIVE
DA_3M_1	NEGATIVE
DA_3M_2	NEGATIVE
DA_3M_3	NEGATIVE
D_2AM_1	POSITIVE
D_2AM_2	NEGATIVE
D_2AM_3	POSITIVE
D_2AM_4	NEGATIVE

Table.9.1 Antibiotic sensitivity assay

ANTIBIOTIC SENSITIVITY PATTERNS (RESISTANT/ SENSITIVE) AS PER CLSI STANDARDS								
SAMPLE NUMBER	**COLISTIN**	**GENTAMICIN**	**TICERCILLIN**	**IMIPENEM**	**CIPROFLOXACIN**	**NETILLIN**	**POLYMYXIN-B**	**AMIKACIN**
DA_2M_1	SENSITIVE	SENSITIVE	RESISTANT	SENSITIVE	SENSITIVE	SENSITIVE	SENSITIVE	SENSITIVE
DA_2M_2	SENSITIVE	SENSITIVE	RESISTANT	SENSITIVE	SENSITIVE	SENSITIVE	SENSITIVE	SENSITIVE
DA_3M_1	SENSITIVE	SENSITIVE	SENSITIVE	SENSITIVE	SENSITIVE	SENSITIVE	SENSITIVE	SENSITIVE
DA_3M_2	SENSITIVE	SENSITIVE	SENSITIVE	SENSITIVE	SENSITIVE	SENSITIVE	SENSITIVE	SENSITIVE
DA_3M_3	SENSITIVE	SENSITIVE	SENSITIVE	SENSITIVE	SENSITIVE	SENSITIVE	SENSITIVE	SENSITIVE
D_2AM_1	SENSITIVE	SENSITIVE	SENSITIVE	SENSITIVE	SENSITIVE	SENSITIVE	SENSITIVE	SENSITIVE
D_2AM_2	SENSITIVE	SENSITIVE	SENSITIVE	SENSITIVE	SENSITIVE	SENSITIVE	SENSITIVE	SENSITIVE
D_2AM_3	SENSITIVE	SENSITIVE	RESISTANT	SENSITIVE	SENSITIVE	SENSITIVE	SENSITIVE	SENSITIVE
D_2AM_4	SENSITIVE	SENSITIVE	SENSITIVE	SENSITIVE	SENSITIVE	SENSITIVE	SENSITIVE	SENSITIVE

Table.9.2 Antibiotic sensitivity patterns (resistant/ sensitive) as per CLSI standards (m.m)

ANTIBIOTIC SENSITIVITY PATTERNS (RESISTANT/ SENSITIVE) AS PER CLSI STANDARDS (m.m)								
SAMPLE NUMBER	**COLISTIN**	**GENTAMICIN**	**TICERCILLIN**	**IMIPENEM**	**CIPROFLOXACIN**	**NETILLIN**	**POLYMYXIN-B**	**AMIKACIN**
DA_2M_1	19.4	35.2	0	52.6	36.2	30.4	18.2	33.4
DA_2M_2	15	24.2	0	21.8	35.8	30	14.2	29
DA_3M_1	16.8	28.4	9.2	53	39.8	33	20	39.6
DA_3M_2	12.8	22.4	19.8	30.2	29.8	25.4	14.2	27.6
DA_3M_3	14.6	29	13	44.2	31.2	29.6	17	29.6
D_2AM_1	12.5	30.4	10.1	24.5	30	30	13.4	25.6
D_2AM_2	16.2	27	13.2	52.2	42	28	23.5	42.5
D_2AM_3	18.6	22	0	20	35.2	34.6	13.5	31
D_2AM_4	15.2	32	14.2	51.5	29.5	30.6	18	31.2

Figure.1 Map of South 24 Parganas showing the collection site-Diamond Harbour (Pal *et al.*, 2015)

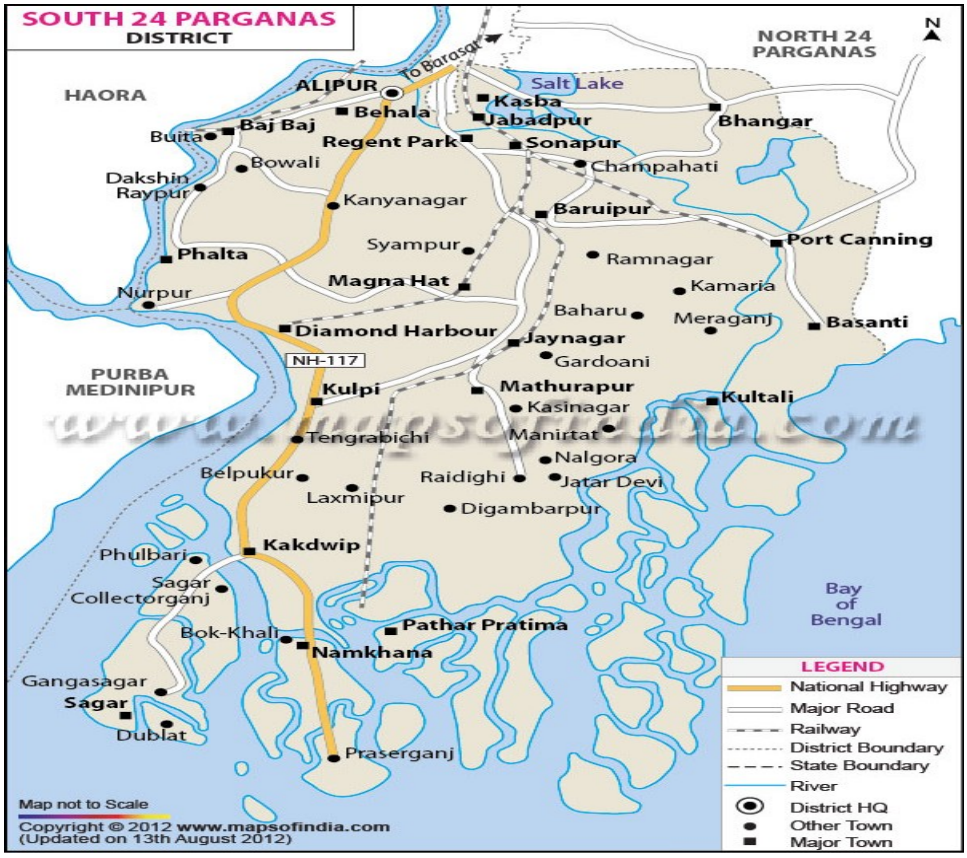
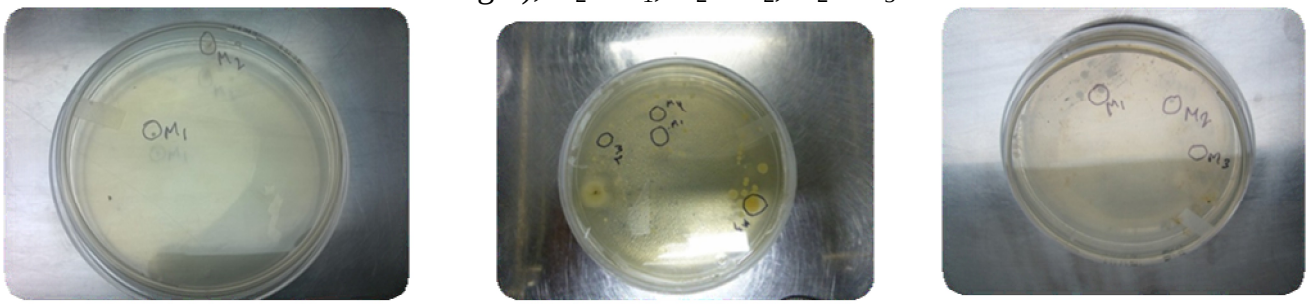


Figure.2 Mophotypes DA_2M_1, DA_2M_2 (upside left) DA_3M_1, DA_3M_2, DA_3M_3 (upside right), D_2AM_1, D_2AM_2, D_2AM_3



& D_2AM_4 (down middle)

Figure.3 DNase production assay of DA_2M_2 (upside left), DA_3M_3 (upside right), D_2AM_3 (down left) & D_2AM_4 (down right)

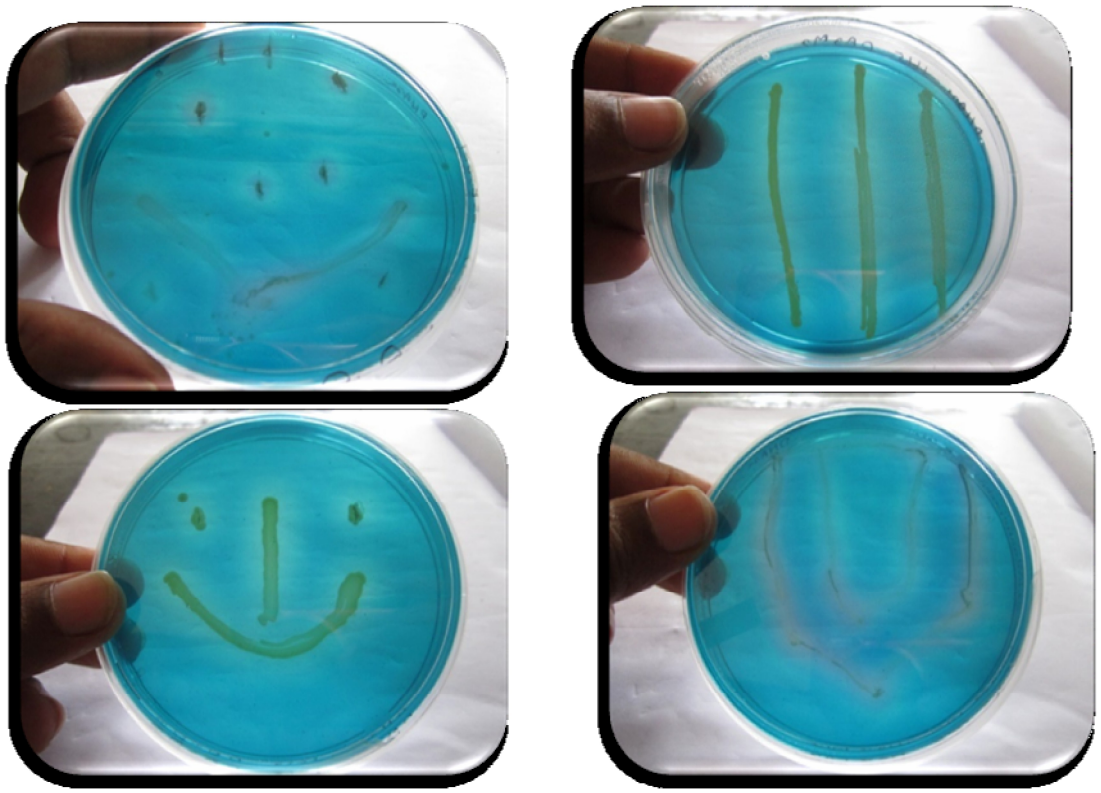


Figure.4 Citrate production of D_2AM

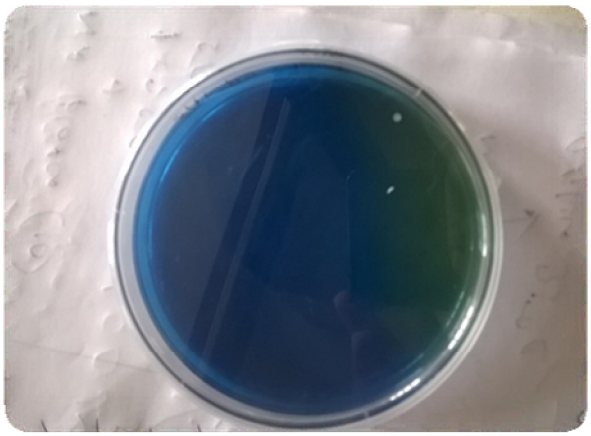


Figure.5 MR test of DA_3M_2 (upside left), DA_3M_3 (upside right), DA_3M_1 (down left) & D_2AM_2 (down right)

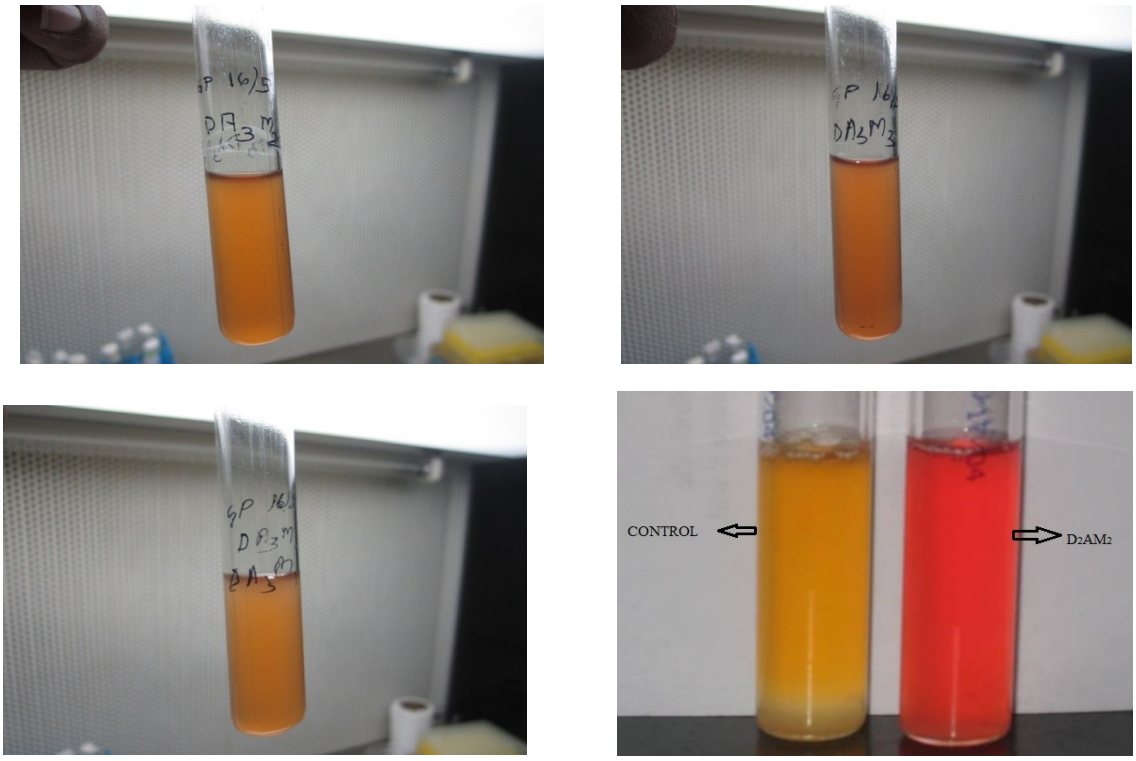


Figure.6 Isolates which show negative result in reaction with Kovac's indole reagent

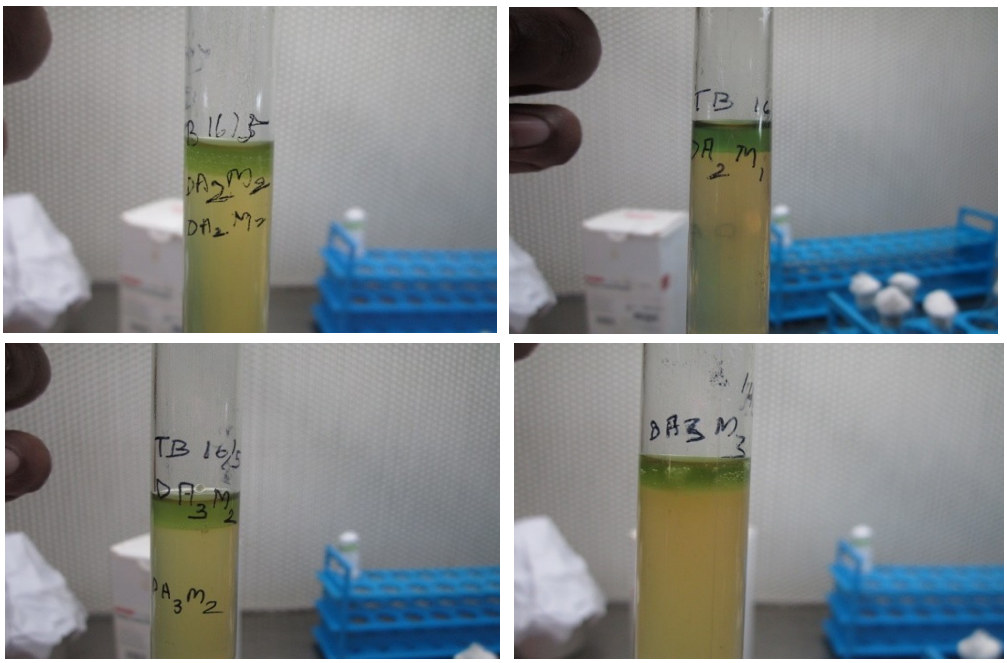


Figure.7 VP TEST DA_2M_2 (upside left), D_2AM_1 (upside right) & D_2AM_3 (down middle)

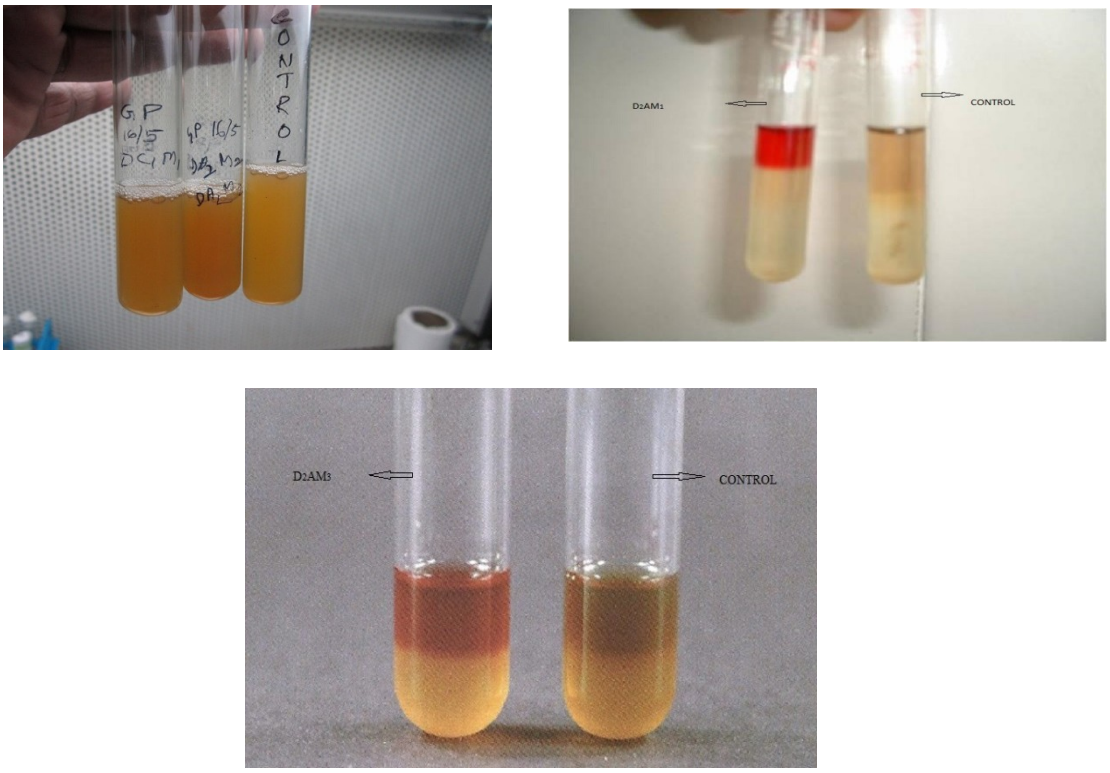


Figure.8 Graph of sensitivity assay

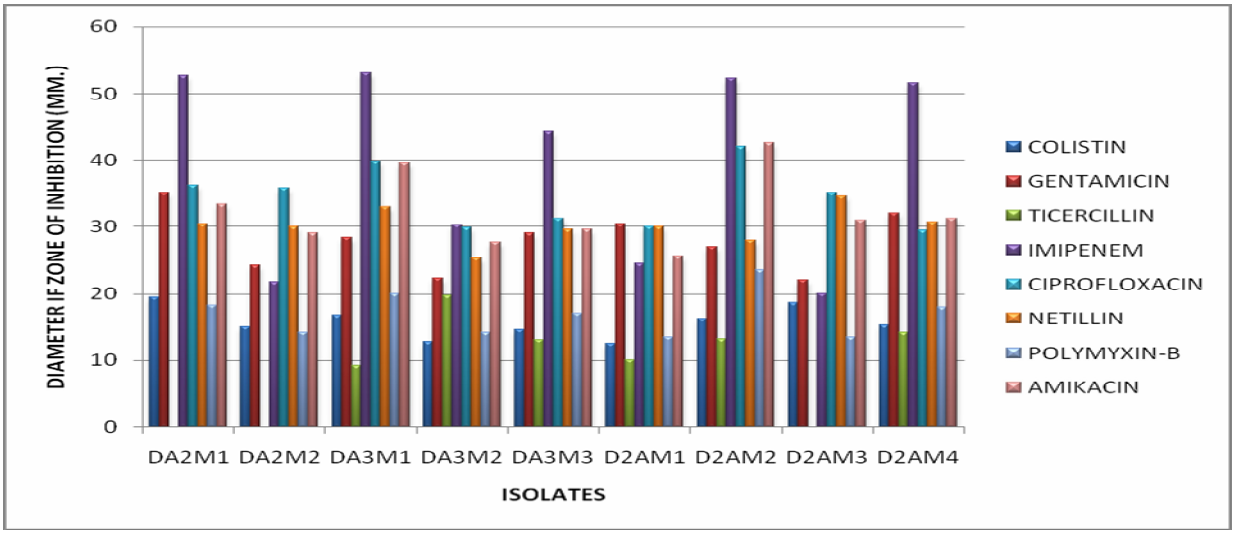
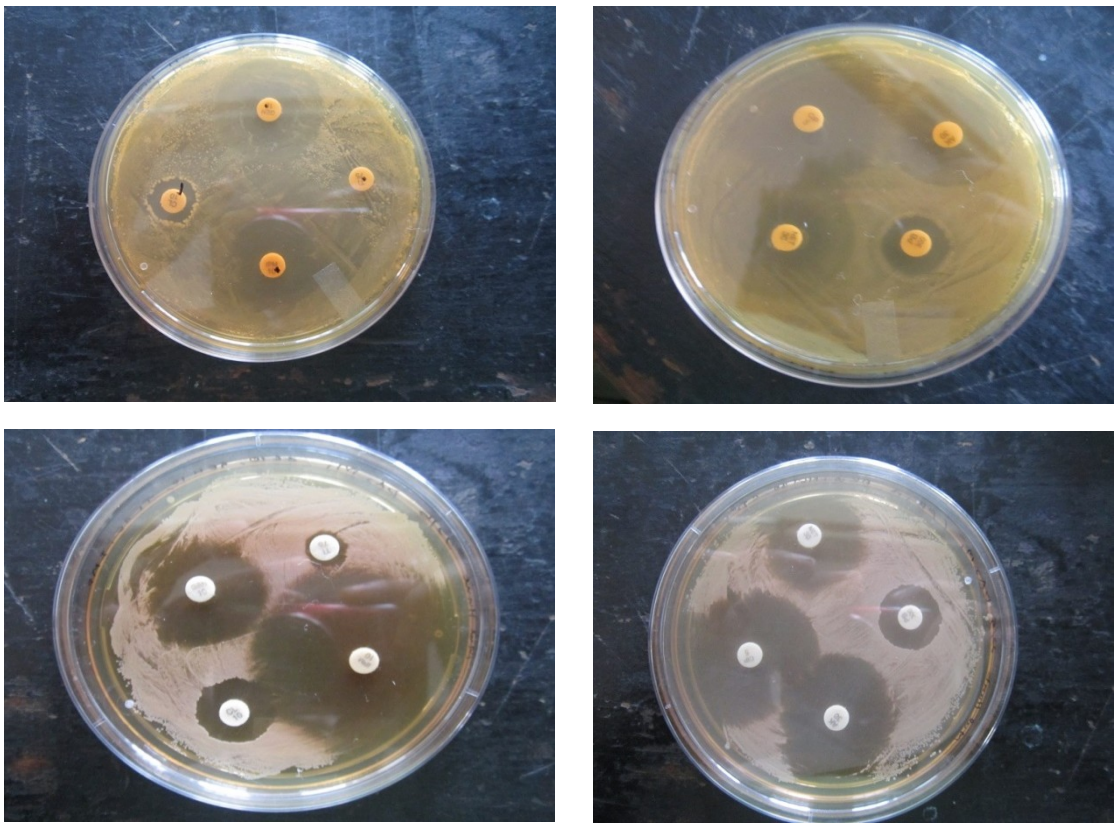


Figure.9 Antibiotic sensitivity assay which show sensitive and resistant zones



Anopheles 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 (Pal *et al.*, 2015). The mid gut bacterial diversity along with the biochemical characteristics is closely associated with the complex potential interaction between the symbiotic microbes and host (Pumpuni *et al.*, 1996). 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 intra-cellularly living endosymbiont bacteria. It can be concluded that the present study on midgut bacterial isolates along with their antibiotic resistance and different biochemical characteristics study may open new windows for better understanding of *Anopheles*-midgut microbiota interaction.

Acknowledgement

The authors are grateful to Swami Shastrajnanada, Honourable Principal and respected Dr. Asit Kr. Sarkar, Head, Dept. of Microbiology, Ramakrishna Mission Vidyamandira, BelurMath, Howrah and Prof. Laltluangliana Khiangte, the Principal, Serampore College, for providing facilities and also for their continuous encouragement to carry out present work

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