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Research Article

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Studies on Midgut Microflora of Aedes Genus Mosquitoes from Badnapur Region (Jalna District), Area of Maharashtra, India

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ABSTRACT

Mosquitoes are responsible insect as a vector to cause various diseases. If not cured on time it goes towards fatality. Majorly attacks of anopheles and aedes genus recently have an impact for causing Malaria, dengue, etc. We focused on aedes which was available in our local areas and easily identifiable with morphological characteristics of larvae and adults. First we collected larvae from storage and sewage water samples. Rearing was carried out in vitro with provision of different flower parts for feeding. All stages of life cycle were observed. For further study of gut Microflora adult stage mosquito dissected and exposed it's gut to nutrient agar medium with saline. Characterization was done with different reagents, media and antibiotics. Amylase production detection assay, Catalase production detection assay, Citric acid utilization assay, Methyl red assay were positive. Phosphate solubilization activity detection assay, Arginine dihydrolase production detection assay, Indole Acetic acid (IAA) production detection assay, Voges Proskauer assay were negative. Isolate was resistant to all antibiotics such as Gentamycin, Kanamycin, Chloramphenicol, Vancomycin and Tetracycline. Gram staining was shown gram negative nature with pink colored colonies.

Keywords: *Anopheles, Aedes, Rearing, Gut microflora, Vector, Antibiotics.*

INTRODUCTION

Mosquitoes are distributed widely. Some species have been spread by human activity. Aedes is a genus of mosquitoes originally found in tropical and subtropical zones, but now found on all continents except Antarctica. The generic name comes from the Ancient Greek aedes, meaning "unpleasant" or "odious". Actually anopheles, aedes, culex and mansonia found in the area of Jalna abundantly (Jaid et al., 2011). Different genus causes several diseases according to their specificity. Mosquitoes are arthropods which are haematophagous vectors cause several diseases like lymphatic filariasis, dengue, malaria, etc. After 2005-06 Dengue and Chikungunya are the diseases widely spread in Marathwada region (Shinde, 2011). Genus Culex is a vector which responsible for disease filariasis. In develoblack ping countries filarial is highly prevalent (Pidiyar et al., 2004).

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Several species of Aedes are invasive. Some species of this genus transmit serious diseases, including dengue fever, yellow fever and Zika virus. The midgut microbiota of mosquito plays important role in vector susceptibility and parasitic or pathogenic interaction (Pal et al., 2015).

Preliminary investigation was done to determine biochemical characterization like gram staining, pattern of growth, detection of enzyme as well as antibiotic susceptibility assay of midgut bacterial isolates of *Culex* (*Culex quinquefasciatus*) in some urban, sub-urban and rural areas of West Bengal (Kumar et al., 2014). The microbiota plays an important role in modulating the mosquito's capacity to sustain Plasmodium infection in anopheles (Dong et al., 2009). *Aedes* mosquitoes are visually distinctive because they have noticeable black and white markings on their body and legs. Unlike most other mosquitoes, they are active and bite only during the daytime. The peak biting periods are early in the morning and in the evening before dusk.

MATERIALS AND METHODS

Collection of Mosquitoes

The mosquito samples were collected by hand catching from Badnapur with GPS location N 19^0 86' 59": E 75^0 72' 50" in winter, in the month of November 2019. Samples from different sources sewage and storage water were of anopheles, aedes and culex. Collected samples kept back in net-house.

Rearing:

Anopheles, aedes and culex kept as in their original source. Feeding is done regularly with flower nectors dipped in water.

Isolation of gut-flora:

During the Midgut bacterial investigations, aedes mosquitoes were sterilized 5 sec. in 70% ethanol and dissected under the microscope.For the isolation of bacteria mosquitos, they were homogenized in 1.5 ml of phosphate buffered saline (PBS), spreaded on Nutrient agar plates (peptic digest of animal tissue 5gms/L, NaCl 5gms/L, Beef extract 1.5gms/L, Yeast extract 1.5 gms/L, Agar 15 gms/L, distilled water 1 L, Final pH at 25^0C ±0.2).

i) Amylase production detection assay

Culture was grown overnight (18-20hrs.) at 35±2°C in nutrient broth and streaked on sterile starch agar medium. Incubation was done for 48 hrs. Colonies developed in petriplates were scrapped with sterile nichrome wire loop. Diluted iodine solution poured on bacterial colonies for 60 seconds. Excess iodine solution was drained off.

Composition of Starch agar medium:

Starch soluble 20 gms/L, Peptone 5 gms/L, Beef extract 3 gms/L, Agar 15 gms/L, Final pH at 25^0C ±0.2.

ii) Phosphate solubilization activity detection assay

Culture was grown over night (18-20 hrs.) at 35± 2°C in nutrient broth and stabbed with the help of inoculation needle into the sterile pikovskaya's agar test tube. Incubation was done at 35± 2°C for 120 hrs.

Composition of pikovskaya's agar medium

Yeast extract 0.5 gms/L, Dextrose 10 gms/L, Calcium phosphate 5 gms/L, Ammonium sulphate 0.5 gms/L, Potassium chloride 0.2 gms/L, Magnesium sulphate 0.1 gms/L, Magnesium sulphite 0.001 gms/L, Ferrous sulphate 0.001 gms/L, Agar 15 gms/L, Final pH at 25^0C ±0.2.

iii) Arginine dihydrolase production detection assay:

Culture was grown overnight (18-20hrs.) at 35± 2°C in nutrient broth and sterile arginine dihydrolase agar medium were prepared and cast stab in sterile culture tubes. Culture was pierced into stab with the help of sterile inoculation needle and incubated at 35± 2°C for 48 hrs. Then 5-6 drops of nessler's reagent was added to the culture tube.

iv) Catalase production detection assay:

Culture was grown overnight (18-20 hrs.) in nutrient broth and streaked on nutrient agar medium. Incubation was done for 48 hrs at 35± 2°C. 2-3 drops of 15% H_2O_2 added on the grown colonies.

v) Indole acetic acid production detection assay:

Culture was grown overnight (18-20 hrs.) at 35± 2°C in nutrient broth with 0.1% tryptophan. The bacterial cells were collected from the culture medium by centrifugation. One ml of supernatant was mixed vigorously with four ml of salkowski's reagent and the absorbance was measured at 535 nm with spectrophotometer.

Composition of salkowski's reagent

150 ml conc. H_2SO_4, 250 ml H_2O, 7.5 ml 0.5 M $FeCl_3.6H_2O$ as per proportion.

vi) Citric acid utilization assay

The culture was grown over night (18-20hrs.) at 35±2 °C in nutrient broth. Sterile simmon citrate agar medium was prepared and cast as stab in sterile culture tube the culture was pierced into stab with the help of a sterile inoculation needle an incubated at 35± 2 °C for 48 hrs.

vii) Methyl red assay

The culture was grown over night (18-20 hrs) at 35±2 °C in nutrient broth. 200 µl culture from overnight grown was inoculated in sterile MR-VP medium in test tubes. Incubation was done at 35±2 °C for overnight in shaking incubator. 4-5 drops of methyl red indicator was added.

Composition of MR-VP medium

Buffered peptone 0.7 gm, glucose 0.5 gm and dipotassium phosphate 0.5 gm per 100 ml.

Composition of methyl red indicator solution

200 Methyl red in 60 ml of ethyl alcohol and made volume upto 100 ml with distilled water. It was stored at 4 °C.

viii) Voges proskauer assay

Culture was grown over night (18-20 hrs) at 35±2 °C in nutrient broth. 200 µl culture from overnight grown was inoculated in sterile MR-VP medium in test tubes. Incubation was done at35±2 °C for overnight in shaking incubator. 1 ml barritt's reagent was added.

Composition of MR-VP medium

Buffered peptone 0.7 gm, glucose 0.5 gm and dipotassium phosphate 0.5 gm per 100 ml.

Composition of barritt's reagent

α-naphthol (1-naphthol) 5gm, ethanol 100 ml.

ix) Antibiotyping

Media Mueller Hinton agar prepared and poured in autoclaved petriplates. Culture was grown overnight (18-20 hrs.) at 35±2 °C in Mueller Hinton broth at 35±2 °C at 110 rpm shaker speed. 200 µl from overnight grown culture spreaded over solidified Mueller Hinton Agar. Plates soaked for 25 minutes at 25 °C in laminar air flow. After soaking, antibiotic discs (Himedia) were used to check the antibiotic sensitivity of isolate. Five different antibiotic discs were used for isolate: Gentamycin, Kanamycin, Chloramphenicol, Vancomycin and Tetracycline. All the experiments were performed in triplicate.

Composition of Mueller Hinton agar medium

Beef infusion 300 gms/L, Casein acid hydrolysate 17.5 gms/L, Starch 1.5 gms/L, Agar 17 gms/L, distilled water 1L Final pH at 25 °C ±0.1.

x) Gram staining of isolate

Gram staining of isolate was carried out following the procedure of Harrigan & MacCance (1976).

1. **OBSERVATION**

Eight biotyping assays were carried out which mentioned in following table 1.

RESULTS AND DISCUSSION

First we isolated flora from mosquito gut in Badnapur region and did several biotyping assays, colony characteristics, antibiotyping and Gram staining. Findings are differentiated in table formats (table 1, 2, 3, 4 & fig. 1). Isolates were cultured on different media such as starch agar medium, pikovskaya's agar medium, arignine dehydrolase agar medium, nutrient agar medium, simmon citrate agar medium and MR-VP medium with different reagents salkowski's reagent, methyl red indicator and barrit's reagent. Antibiotyping was carried out in Mueller Hinton agar medium with different antibiotics such as Gentamycin, Kanamycin, Chloramphenicol, Vancomycin and Tetracycline.

Table 1 shows biotyping assays. Amylase production detection assay carried out on starch agar medium with addition of iodine solution. Iodine shows blue black colour in the presence of starch. Bacteria shown clear zone around the growth produce the exoenzyme amylase. No zone of clearance in pikovskaya's agar medium around the growth of bacteria so test was negative. Arginine dihydrolase test was negative due to colour change from purple to yellow but not reversed back to purple. During catalase production detection assay bubble formation was observed after adding H_2O_2 shows breakdown of it into O_2 and H_2O. The test was positive. Indole acetic acid production detection assay was negative. In citric acid utilization assay test was positive due to change from original green to blue colour of the medium. This indicates the alkaline carbonate and bicarbonates produced as by-products of citrate catabolism which raise the pH of medium above 7.6. Methyl red assay shows red colored ring after addition of methyl red indicator, the test was positive due to metabolism of pyruvic acid to other acids which lowers the pH of the medium to 4.2. Voges proskauer assay was negative.

Table 2 shows the colony characteristics in which isolate with irregular colony shape, large size, opaque opacity, glossy texture, yellowish color and rough morphology surface.

Table 3 shows isolate's resistance to all antibiotics which were used in the form of discs. Mean zone of inhibition was more in Tetracycline while no cone of inhibition was in Vancomycin.

Table 4 After Gram's staining colonies turned into pink colored, it indicates isolate of mosquito midgut was Gram negative. Other characteristics were also observed in which isolate was with rod shape and round edge morphology, small size and occurs individually.

Mosquitoes were collected and rearing was done conventionally. There were much more difficulties like damage to organs like legs, abdomen, wings, etc. while collecting.

It has been reported that midgut microflora share their habitat during the larval development (Yadav et al., 2015). Earlier studied shown that the midgut microflora of mosquitoes significantly affects on the digestion, metabolism, and immunity of their hosts (Yadav et al., 2016). Anopheles genus was identified by observing the terminal segments of generalized mosquito larva (Snell, 2005). Different genera of mosquitoes were morphologically studied according to several keys (Rattanarithikul, 1982). A number of factors affect the vector competence of midgut microflora mosquitoes (Tiawsirisup et al., 2018). In the present study, we observed pecten on the basis of which we differentiated their genus when the mosquitoes left in net much differences in larval growth due to feeding irregularities because of the growth in the environment like water source (fresh water, storage water or sewage water).

Anopheles mosquitoes prolong life cycle in fresh tap water due to deficient source of nutrients which required for their growth. Juice from cereals artificially we can prepare for their growth.

Experiments were carried out in triplicate to avoid chance of errors in aseptic conditions. We reviewed research articles in which they got 3-4 types bacteria, where in our study we got only one type of bacteria this can be just because of growth conditions which were provided for the growth of mosquitoes. The acquisition and structure of bacterial communities in mosquitoes of midgut microflora was reported as Coon et al. (2014). We had prepared M_9 medium on which suspensions with soil were spread to isolate *Bacillus thuringiensis*. Application of culture media was resulted good output of microflora of the field caught specimens was reported Chavshin et al. (2012). After isolation we prepared serial dilutions in distilled water to detect larvicidal activity.

Table 1: Biotyping assessment of isolated microflora

Sr. no.	Biotyping assays (Test)	Results (Positive and Negative)
1	Amylase production detection assay	+
2	Phosphate solubilization activity detection assay	-
3	Arginine dihydrolase production detection assay	+
4	Catalase production detection assay	+
5	Indole Acetic acid (IAA) production detection assay	-
6	Citric acid utilization assay	+
7	Methyl red assay	+
8	Voges Proskauer assay	-

The + signs indicate positive while - signs indicate negative results.

Table 2: Colony characteristics of isolates

Colony Parameters	Colony Shape	Colony Size	Colony Opacity	Colony texture	Colony colour	Colony Surface morphology
Colony Characteristic	Irregular	Large	Opaque	Glossy	Yellowish white	Rough

Table 3: Antibiotic resistance assessment (*Aedes* gut bacteria isolate from Badnapur)

Name of Antibiotic	Zone of inhibition (in cm)	Mean Zone of Inhibition (in cm)	Resistance type
Gentamycin	0.8 0.5	0.6	Resistant
Kanamycin	0.5 0.5	0.5	Resistant
Chloramphenicol	0.6 0.4	0.5	Resistant
Vancomycin	0.0 (No zone of inhibition) 0.0 (No zone of inhibition)	0.0	Resistant
Tetracycline	0.9 0.9	0.9	Resistant

Table 4: Characteristics of Isolate after Gram Staining

Cell Parameters	Cell Gram Character	Cell Morphology	Cell size	Cell Association
Cell characteristics	Gram negative	Rod shaped with round edge	Small	Occurs individually

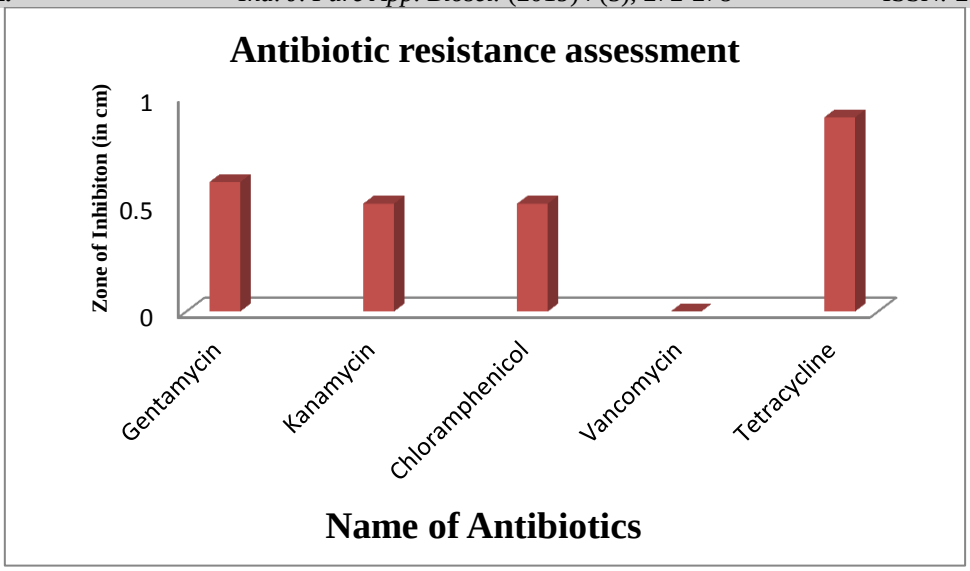

Fig. 1: Antibiotic resistance assessment (*Aedes* gut bacteria isolate from Badnapur).

CONCLUSION

It was concluded that the Amylase production detection assay, Catalase production detection assay, Citric acid utilization assay, Methyl red assay were positive. Phosphate solubilization activity detection assay, Arginine dihydrolase production detection assay, Indole Acetic acid (IAA) production detection assay, Voges Proskauer assay were negative. Isolate's nature was Gram negative with resistance to antibiotics used. It was possible that by the present type of studies, mosquitoes were very well identified earliest and the necessary action will be taken for destruction of mosquitoes**.**

FUTURE PERSPECTIVES

The collection and gut flora maintaining of mosquitoes was challenging in case of rearing we can use advance methods to collect and rearing of mosquitoes. We can study different genus for gut flora in future. According to priority anopheles is major in transferring diseases like malaria.

Susceptibility of Mosquitoes to Dengue Viruses in accordance with microbial gut flora of *Aedes aegypti* (Mourya et al., 2002) can also be studied with other genus which is present in Jalna region.

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