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Isolation and biochemical identification of Mid Gut microbiota of wild caught and lab reared *Anopheles Stephensi* and their possible role in disease transmission

Ankita Kumari[1], Arti Prasad[2]
[1] Research Scholar, Department of zoology, University Collage of Science, Udaipur, Rajasthan, India
[2] Professor and Head, Department of Zoology, University Collage of Science, Udaipur, Rajasthan, India

Abstract
For description the midgut microbiota diversity to find the seeker bacterial for the transmission of malaria, the microbiota of wild adult Anopheles stephensi mosquito and adult lab-reared Anopheles stephensi mosquito midgut which were collected from southern udaipur was studied using a culture dependent, biochemical-based technique. Gram staining and specific media were used for the identification of Gram-positive and Gram–negative bacterial colonies. Isolated and selected colonies were identified by different physiological, biochemical tests and techniques. Eighteen species in 9 genera in which six Gram-negative: Aeromonas, Myroids, Pseudomonas, Klebsiella, Enterobacter and Shewanella and three Gram-positive Microbacterium, Enterococcus and Rhodococcus bacterial colonies were isolate and identified in the mid gut of the wild cough anopheles stephensi mosquito. 12 Gram-negative species in three genera including Pseudomonas, Myroides and Aeromonas were isolated and identified from lab-reared anopheles stephensi mosquitoes. Pseudomonas and Aeromonas with a frequency of 51% and 14% field caught and in lab reared midgut with a frequency of 54% and 20% were the most common midgut symbionts. Three genera have been isolated from both wild and lab reared mosquitoes were Pseudomonas, Aeromonas and Myroides. Fastidious growth of bacteria shown in inexpensive media, which were Gram negative, and found dominant in both wild and lab reared anopheles stephensi mosquitoes and presence in other malaria vectors.

Keywords: anopheles, bacteria, biochemical characteristics, midgut, lactobacillus, bacillus, staphylococci

Introduction

Among approximately 60 Anopheles species anopheles stephensi is one which is painstaking in malaria disease communication. Anopheles also known as Asian malaria vector. Anopheles stephensi is a key vector for malaria transmission not only in India but also all over in Asia. Plasmodium falciparum have highest vulnerability of this anopheles species and the global malaria control and elimination programmes might be challenge by its lenience in the future.

Vector-born diseases creates greatest challenges for global health. At present there is decreases in malaria illness and mortality have noticed at reversed level, and viral diseases which including dengue and chikungunya fever and Zika, are remain largely at unchecked level [13]. While research for mass drug administration and the vaccine development are continue. Vector control strategies remains the most significant and cost-effective way to protect populations from malaria epidemics. However, current threatening are gains in resistance development in insecticides [14] and due to this number of research efforts has raised to develop genetic strategies to control malaria transmission [12].

All aspects related to microbial relationships, from pathogenic to obligate mutualism signifies by the gut microflora. Study of insect–microbial pathogen associations and produce microbial insecticides comes after study on insect-bacteria symbiosis. The current research into endosymbionts such as Wolbachia and Buchnera spp. has encouraged the focus away from the pathogenic relationship and re-emphasized the extent to which microbes have integrated with insects.

Relationships between insects and gut bacteria or pathogens are only deliberated in the framework of the ability of the microbiota to prevent pathogen growth. The consequence of the mid gut bacteria is discussed in the context of rising our understanding of symbiotic relationships and multitrophic associates between insects and plant or animal hosts, and in developing new strategies for controlling insect pests. Using culturing methods some few studies have been performed to investigate bacterial species in field-collected Anopheles mosquitoes. Gonzalez-Ceron *et al*. isolated the colonies which were belonged to Enterobacter amnigenus, Enterobacter cloacae, Enterobacter sp., Serratia marcescens, and Serratia sp. from Anopheles albimanus mosquitoes caught in southern Mexico. To identify bacterial strains for a paratransgenic mosquito, we conducted a screen for cultured midgut bacteria from wild-caught Anopheles stephensi and lab reared Anopheles stephensi mosquitoes.

At this present time there is recent attention in the use of microorganisms as biological control agents of vector-borne diseases. Microorganisms which are connected with vectors could exert a direct pathogenic effect on the

host by interfering with its reproduction system or could reduce vector competence [10]. The bacteria in the midgut can be acquired both trans-staidly and through contaminated sugar solutions and bloodmeals in laboratory – reared mosquitoes. However, the origin of the midgut bacteria, are still unknown in wild populations of mosquitoes [21, 8]. To identify the organisms that play significant roles in the conservation of these communities an understanding of the microbial community structure of the mosquito midgut is essential, which will enable us to understand. We have conducted a screen for midgut bacteria from lab-reared and wild-caught Anopheles stephensi mosquitoes using "culture-dependent" approach to understand the bacterial diversity and to isolate and identify bacteria for a paratransgenic mosquito.

Experimental

Collection and characterization of An. Stephensi larvae and adult mosquitoes from field

Larvae and adult of Anopheles stephensi were collected from Udaipur district, an urban and peri-urban, mountainous with many water bodies and river. By using the standard dipping technique (350 ml dipper) larvae were collected from larval habitats and adults were captured from pit shelters and human residences with the help of aspirator. The adult mosquitoes were transferred alive to the laboratory of Laboratory of Public Health-Entomology (LPHE). After that mosquito were identified to species level by using standard morphological key. Adult females were selected for analysis of wild midgut microbiota. Larvae of An. stephensi were kept in laboratory for pure-line culture for further midgut microbiota analysis of lab-reared anopheles vector.

Remaining Field-collected mosquitoes and larvae were maintained at the standard LPHE insectory condition of 27±1°C temperature, 70–80% humidity with 12:12 h light and dark cycle. The mosquitoes were allowed to feed from membrane blood feeder. After 3–4 days post-blood meal through membrane feeder, eggs were collected in ovitraps. Eggs shifted to the rearing trays which were containing dechlorinated water for larval emergence.

Larvae were maintained in the rearing trays at a density of ~200 larvae/l of water and fed with a mixture of yeast power and dog biscuit in a ratio of 3:2. Emerging pupae after 10–12 days were collected daily and kept in mosquito cages for adult emergence. Emerged adults were maintained on sterile 10% glucose solution. Adult female anopheles stephensi mosquitoes generations from F6–F8 generations were used for the midgut bacterial studies.

Before the discussion of mosquitoes, the surface of the adult anopheles stephensi were sterilized with 70% ethanol in a sterile condition (LAF). Under sterile conditions in Laminar air flow anopheles were dissected separately and the midguts were mashed and suspended in 250 ml of Nutrient broth (NB). A 100 µL amount of the contents was serially diluted up to 10−3 and then dilution 10^9, 10^6, 10^4, 10^2 were plated onto the following four media those were (1) Mannitol Salt Agar (MSA),(2) Nutrient agar (NA),(3) MacConkey agar, (4) Eosin methylene blue agar (EMB), and after the plating incubated at 28 ± 2 ◦C for 24–48 h. During the entire procedure the sterility of all reagents were checked by using the control plate of all media with each dilution. For the isolation and identification of single purified colonies of the bacteria, continuous sub-culture of every grown bacterial colony were performed individually repetitively until the pure isolated colonies. The purified isolated single colonies of the pure bacterial culture later were used for physiological identification, morphology identification, biochemical analysis.

Selection of mosquitoes and collection midgut samples

For midgut microbiota analysis 1-2 days old mosquitoes were selected which were newly emerged. Dissections of mosquitoes were processed under sterile conditions after anesthetized adult. For dissections firstly mosquitoes were surface sterilized with a 70% ethanol solution, and then rinsed thrice in sterile phosphate-buffered saline (PBS). After then under a stereo microscope (10× magnification) using sterile forceps midguts were carefully removed. And then crushed individually in 50 µl of sterile PBS using a single-use pestle.

Culture dependent midgut bacteria isolation

The Nutrient agar, EMB, Mannitol salt agar and MacConkey agar were four media which were used in this study. Each agar which were used in this study have their specific characteristic for specific bacterial growth. Specific characteristic of each agar used in this study shown (Table:1) respectively: The MacConkey agar (European Pharm.) used as a selective culture medium for the isolation of Gram-negative bacteria, especially for the family of Enterobacteriaceae. Each midgut homogenate was plated on nutrient agar using the spread plate dilution method to obtain isolated bacterial colonies. The MacConkey agar medium allows variation between lactose-fermenting and non-lactose fermenting Gram-negative bacteria; lactose fermenting bacteria appear as red/pink coloured colonies whereas those non-fermenting bacteria produce white/colorless colonies. For the isolation of pure bacterial isolates, bacterial colonies with distinct morphologies, colors and margins were picked and sub-cultured into Nutrient Agar (NA) agar.

Isolation of bacterial colonies and morphologically characterization- Serially diluted midgut suspension streaking was done on Nutrient agar (NA) plates and put in incubator for incubation at 37°C for 48 h.

For bacteria preservation all bacteria were re-streaked in deep stick culture. Morphologically identification of bacteria was done after examined using visual and microscopic investigation. Confirmation isolation of all bacterial isolate were done by using classical morphologically and biochemical test. Gram-staining, oxi/ferm

tests and using selective cultivation media were done. For confirmatory identification of isolated colonies all biochemical and phenotyping methods were compared for better results.

Table 1: Selective media that were used for culture the bacterial colony

Type of media	Selective for bacteria
MacConkey agar (DMP/NICD, South Africa; cat no. DMPA0315)	Gram-negative non-fastidious
NA (Nutrient Agar)	A variety of fastidious bacteria
Mannitol salt agar (DMP/NICD, South Africa; cat no. DMPA0316)	Gram +ve bacteria
Levine's formulation (or Eosin methylene blue) / EMB agar	Gram –ve bacteria

Biochemical tests used for characterization-
Preliminary Identification (see table-2)

Table 2

Grams staining	Spore staining	Catalase test	Oxidase test

1. Biochemical Analysis

Table 3

1	**Indole**
2	Methylene Red
3	Vogas Proskauer
4	Citrate
5	Urease
6	H_2S

Result: Colony Characteristics of Each Isolates after Isolation (See table – 4)

Table 4

Colony parameters	**Colony characteristics**	
	Wild	**Lab reared**
Shape of the colony	Round	Circular diffused
Size of the colony	Small	Large
Opacity of the colony	Translucent	Opaque
Texture of the colony	Glossy	Glossy
Colour of the colony	Light Yellow	Yellowish white
Surface morphology of the colony	Smooth	Smooth

Gram Staining results of Each Isolates After Gram Staining procedure
The results of Gram stain of each isolates was observed/determined after Gram staining. The result of gram staining are listed on following table:

Table 5

Cell parameter	**Cell characteristics**	
	Wild	**Lab reared**
Gram character	Gram positive	Gram negative
Morphology	Rod shaped with round edges	Rod shaped with round edges
Size	Very small	Small
Association	Occurred individually	Occurred in chains

List of bacterial phylum associated with *Anopheles stephensi* mosquitoes
Phylum: Actinobacteria

Table 6

Genus	**Family**	**Class**	**Pathogenic**
Microbacterium	Microbacteriaceae	Actinobacteria	Nosocomical pathogen
Micrococcus	Micrococcaceae	Actinobacteria	Non-pathogenic
Rhodococcus	Nocardiaceae	Actinobacteria	Yes-pneumonia
Actinomyces	Actinomycetaceae	Actinobacteria	Yes- gastrointestinal

Phylum: Bacteroidetes

Table 7

Genus	Family	Class	Pathogenic
Myroides	Flavobacteriaceae	Flavobacteriia	Yes (uncommon)

Phylum: Firmicutes

Table 8

Genus	Family	Class	Pathogenic
Bacillus	Bacillaceae	Bacilli	Non-pathogenic
Clostridium	Clostridiaceae	Clostridia	Yes-food intoxication pathogen
Enterococcus	Enterococcaceae	Bacilli	Yes-nosocomial pathogen
Lactobacillus	Lactobacillaceae	Bacilli	Non-pathogenic
Staphylococcus	Staphylococcaceae	Bacilli	Variable according species
Streptococcus	Streptococcaceae	Bacilli	Yes-human opportunistic pathogen

Phylum: Proteobacteria

Table 9

Genus	Family	Class	Pathogenic
Aeromonas	Aeromonadaceae	Gammaproteobacteria	Yes-gastroenteritis
Agrobacterium	Comamonadaceae	Beta-proteobacteria	Opportunistic pathogens
Enterobacter	Enterobacteriaceae	Gammaproteobacteria	Yes-eye & skin infection
Shigella	Enterobacteriaceae	Gammaproteobacteria	Yes-diarrhea
Pseudomonas	Pseudomonadaceae	Gammaproteobacteria	Yes-urinary tract & respiratory system infection

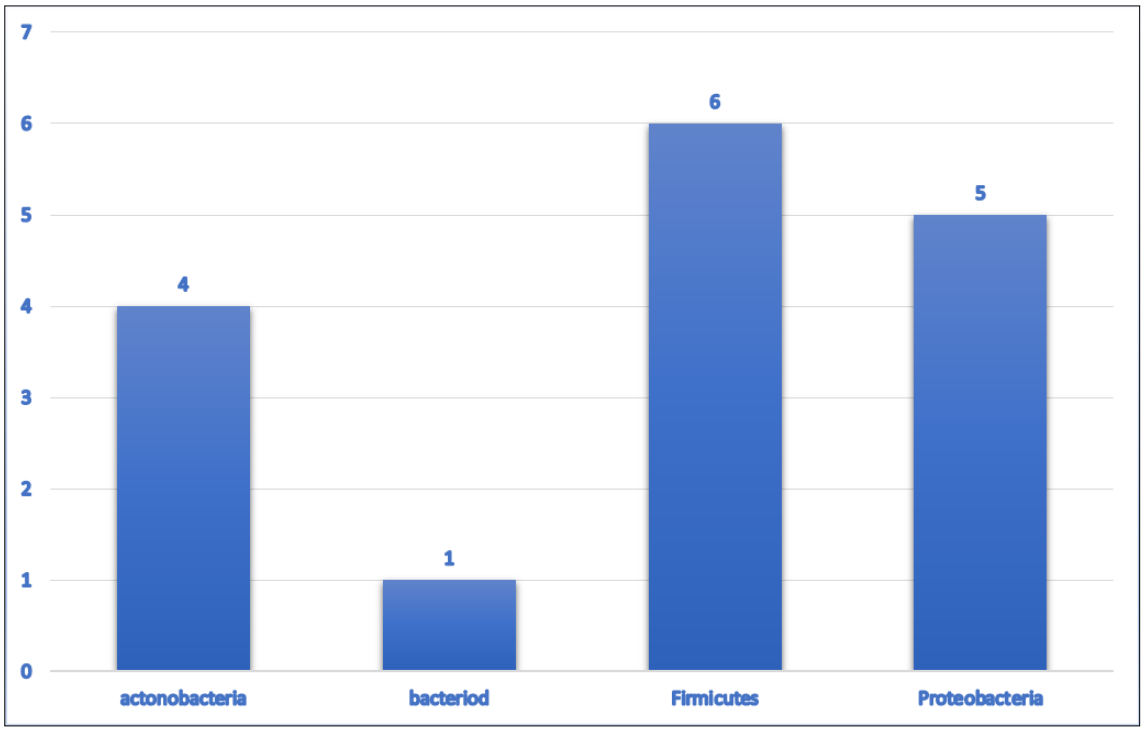

Fig 1: Graphical representation of identified bacterial group, X axis- Phylum Y, axis-identified group number

These above findings provide inclusive information about characters of colony, gram staining characteristics and biochemical analysis of midgut microflora of *Anopheles* mosquitoes which were collected from field area of Udaipur and lab reared.

In this study different capability for the bacteria growth shown by used different culture media (MSA, MCA, NA, and EMB). NA provided fastest growth condition for mostly bacterial colonies, some species can grow on certain substrates and some cannot grow or, possibly show weak or slow growth in other than NA media. Myroides, Enterobacter, Aeromonas and Pseudomonas cultivable species were isolated from NA (Nutrient agar) media. After isolation and purification procedures by using dilution and repeated subcultures results analysis of

colony numbers showed that more than half percentage of the bacterial colonies from the midgut of wild Anopheles stephensi were belongs to Pseudomonas and the remaining colonies were belongs to Exiguobacterium (14 percentages), Aeromonas (7 percentages), and Kocuria, Klebsiella, and Microbacterium (4 percentages) shown descending oreder. Pseudomonas and Aeromonas genera showed frequencies that was more than 54% and 20% respectively, were the most common symbiotic bacteria in lab reared mosquito.

After the examination of the isolated and identified bacteria from adult midguts showed that three genera (belongs to Pseudomonas, Aeromonas and Myroides) were found from both wild and lab reared mosquitoes mid gut. From lab reared mosquitoes midgut Pseudomonas, Aeromonas, Enterobacter and Myroides genera were isolated but Klebsiella, Shewanella and Microbacterium were isolated from wild anopheles stephensi.

The above experimental studies revealed the presence of some groups which were the most commonly found bacteria like Pseudomonas, which containing of the highly identical (99–100% similarity). Both wild and lab reared Anopheles Stephensi mosquitoes midgut have pseudomonas species/strain.

Discussion

In this present study 16 bacteria species were isolated and identified on the basis of physiologically and biochemically identification in the midgut of adult Anopheles stephensi. After analysis the more numbers of bacterial species isolated and identified in this study might be attributable to the origin of the samples (i.e. wild) as well as after using various culturable media for bacterial growth. There is less bacterial growth because laboratory conditions may limit bacteria acquisition at adult stages [4, 6], this resulting in great decrease or nil cultivable bacteria in the mosquitoes midgut [18].

Using of more then one culture media for bacterial growth in this study resulted in a better representation of midgut microflora of the field-caught and lab-reared adult Anopheles stephensi. The species number found in the field cough was more as that present in the lab-reared, may be because of limited laboratory conditions. As previous studies indicate that significant variation occurs due to different habitat shown the effect of environmental habitation of wild caught and lab-reared mosquitoes in aquatic and earthly ecologies respectively [20, 18]. Mostly species found after this research study have already been reported as normal dwellers of mosquitoes midguts flora [5, 15, 11, 22, 16].

Here in present study done on anopheles stephensi midgut most frequently isolated and identified bacterial colony was Pseudomonas spp. However, this is disparity with earlier studies that was reporting Serratia sp. in Anopheles stephensi midgut [16], Bacillus spp. Were reporting in Anopheles arabiensis mosquitoes [11], and Enterobacter spp. was reported Anopheles albimanus as the prominent species [5]. So these reproduces the probable effect of biogeographical factors on the variation of different bacteria species in each region. So we can make conclusion that among the innovative tools for the control of vector-borne diseases, much more importance has increased due to use of microbial symbionts to reduce vector competence in this field of vector control. To identify the midgut microflora of Anopheles stephensi this present research study was intended, and recognize the possible effect of total mosquito-microflora on the expansion of rodent malaria parasite in An. stephensi–P. yoelii model.

Conclusion

However choosing a bacteria/bacterial strain for modification in direction to shorten the life span or interfere with malaria parasite of the malaria vector mosquitoes have several issues those should be resolved for this. We can propose some species which related to pseudomonas for Para-transgenesis program of Anopheles Stephensi because it objecting all required features for appropriate candidate bacteria for Paratransgenesis. However, the above-mentioned issues related to vector control strategies should be addressed to evaluate the possible use of the applicant bacteria.

For scheming communication blocking approaches to control the vector-borne diseases this is very important to understand the natural composition of microbiota of the vectors and its connection with disease communication in the environment.

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