Effect of Gut Microbes from *Eyprepocnemis alacris alacris* (Serv. 1838) against *Culex quinquefasciatus* Say- An Ecofriendly Approach

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Abstract Mosquitoes are on focus worldwide because of its role as vectors for devastating parasites and pathogens causing a threat to millions of people worldwide. Vector-borne diseases in mosquitoes are transmitted by females. However, ineffectiveness of insecticides increases the resistance in mosquitoes. The administration of new vector control strategies against the mosquitoes needs the implementation of microbial insecticides. In this study was commenced to determine the mortality of larvae using gut bacteria as of *Eyprepocnemis alacris alacris* (Serville, 1838) conflict to larvae laboratory conditions to identify an effective bacteria. The results indicated the existence of larvicidal activity in 106 isolates and were found to appear strongly effective activity in three isolates against *Cx. quinquefasciatus*. Cent per cent mortality was observed from each of the secondary metabolites. The recorded values of LC₅₀ were 6.66, 7.13 and 7.64ppm and LC₉₀ has been 115.90, 205.73 and 242.59 ppm respectively. The sequencing and phylogenetic analysis confirmed that these 3 isolates were identified as *Klebsiella pneumoniae*, *Enterobacter xiangfangensis* and *Ochrobactrum intermedium* respectively. Our results showed high larvicidal potential effect against to these bacteria. Outcome of these work highlight the alternative use of synthetic chemical pesticides to control mosquitoes.

Keywords *Culex quinquefasciatus*, Larvicidal Activity, *Klebsiella pneumoniae*, *Enterobacter xiangfangensis*, *Ochrobactrum Intermedium*

1. Introduction

Mosquitoes are one of the major nuisances found more than 30 million years spreading epidemic and disease to humans and their helpful resources like animals globally

[1]. Mosquitoes belong to arthropods and they transmit parasites and pathogens through their piercing and sucking type of mouthparts to human beings [2]. Mosquitoes are expected to convey the ailment more than 700 million people per annum; bites are regularly an annoying nuisance, but still cause some diseases like malaria, dengue fever, yellow fever, etc., every year [(James 1992)].

Cx. quinquefasciatus Say is a cosmopolitan mosquito vector of lymphatic filariasis and Japanese encephalitis. It causes millions of deaths every year, especially in India and Africa [3, 4]. Human filariasis caused by *Wuchereria bancrofti* is a major public health hazard and remains a challenging socioeconomic problem in many of the tropical countries [5]. It is widely distributed in tropical zones with around 120 million people infected worldwide and 44 million people having common chronic manifestation [6] and has a very low control priority [7]. These filarial nematodes are usually characterized by progressive debilitating swelling at the extremities, scrotum, or breast (elephantiasis) in an infected individual [8].

Vector control is a very important part of the global strategy for the management of mosquito-borne diseases and insecticide application is the most important component in this effort. The main tool being used to combat mosquitoes are conventional pesticides but the application of synthetic pesticides can cause health problems and adversely affect the environment [9, 10]. The resurgence of mosquito-borne diseases is due to the development of insecticide resistance and the drug resistance of pathogens [11]. A large number of studies have shown that multiple, complex resistance mechanisms such as, increased metabolic detoxification of insecticides and decreased the sensitivity of the target proteins or genes are likely responsible for insecticide resistance. However, no comprehensive understanding of the resistance mechanisms or regulation involved has been yet been

developed [12].

Consequently, synthetic chemical pesticides should be altered by effective and safe natural insecticides such as plant-derived essential oils, extracts and their compounds [13-17]. Insect-bacterial symbioses are widespread in the environment [18] and antibiotic-producing bacterial symbionts are often used to protect the host and/or their resources [19]. So far the most successful bio insecticide used, in order to combat mosquito larvae are *Bacillus thuringiensis* [20, 21].

Most of the agricultural insects act as pests and are responsible for immense losses in crop plants [22]. Locusts and grasshoppers (Orthoptera: Acridoidea) are among the most perilous pests to tree seedlings and agricultural crops [23]. Synthetic insecticides have relied on traditionally for control of grasshoppers and locusts [24]. However, the high costs of emergency control and eco-friendly management and growing awareness are expanding the demand for biological control [25]. However, symbiotic bacteria residing in the gut of *Eyprepocnemis alacris alacris* also introduce a promising solution as a biocontrol agent for pest control management.

Microbial colonization in an insect gut is due to distinctive environments and provides many beneficial services to their hosts [26]. Symbiotic bacteria exist in the entomopathogenic nematodes also bring as a biocontrol agent for pest control management [27]. In the natural and human-impacted ecosystems insects and their gut microbial communities regarded as the intermediary of biogeochemical cycles [28, 29].

The current study was conducted for laboratory assessment of the mosquito larvicidal activity of *E. alacris alacris* (short-horned grasshopper) gut microbes against early fourth instar larvae of *Culex quinquefasciatus*.

2. Materials and Methods

2.1. Compilation of Insects

Short-horned grasshoppers, *Eyprepocnemis alacris alacris* (Serville, 1838) (Orthoptera: Acrididae) have been compiled from campus area of Periyar University in Salem (Latit. 11.650 N: Long. 78.16E), Tamil Nadu, India. The environmental conditions of the study area for example the temperature; humidity and precipitation were 27°C, 86% and 60% respectively. The adult insects were collected using the sweep net on grasses; bushes and other crop fields such as paddy, sugar cane and maize field etc., and fetched to the laboratory in polypropylene containers [30].

2.2. Isolation and Characterization of Cultivated Bacteria from Insect Digestive Tract

The collected insects were surface sterilized with iodine

and 70% ethanol, further to remove the traces of ethanol insect was cleaned with sterile water. According to the modified procedure of [31], twenty (20N) of adult insects cooled and dissections were performed on ice [32]. The dissected gut tissue was washed in IX phosphate-buffered saline (PBS; 150mM NaCl in 20mM Na⁺/K⁺ phosphate buffer, pH 7.4) and was pooled into microcentrifuge tubes with 100µl of PBS [33]. Furthermore, 1ml of gut extracts was homogenized in 10mM PBS in a Ten Broeck Homogenizer [34]. The homogenates have been vortexed for 10 sec to detach the cells of bacteria from the suspension of gut. Gut extracts have been stored frozen at -20°C. The serially diluted gut suspension was plated on the respective media and pro to incubate for 24hrs at 37°C [35]. Each colony was purified through continuous streaking on agar plates.

3. Test for Mosquito's Larvicidal Activity

3.1. Mosquito's Culture

Larvae of *C. quinquefasciatus* were collected as of the leftovers of sluggish water in various places of Periyar University, Salem. Colonization and maintenance of the larvae for generations have been continued by using the procedure [36, 37] to avoid external contaminants. To avoid the toxicity due to scum formation in water it was changed every day.

3.2. Larvicidal Analysis

Modified method was used to evaluate the devastation of larvae [38]. Early IV instar of 25 *Cx. quinquefasciatus* larvae have been emancipated in 1.0 ml of microbial cultures containing 249 ml of distilled water at different concentrations. A proper distilled water control has been maintained along with the ten replicates of all concentrations. The exposure phase subsequent to 24 hrs fatality and endurance rate have been documented. Percentage of fatality of larvae in each treated concentration was calculated by motile larva were combined with control group larvae in all replicates. Confirmation of the fatal larvae in the experiment was done by perplexing the cervical region [39]. Moribund larvae were those incapables of rising to the surface (within a reasonable period of time) or showing the characteristic diving reaction when the water was disturbed.

3.3. Statistical Analysis

The LC₅₀ and LC₉₀ values of larval fatality in each concentration of trials have been calculated by applying EPA Probit analysis programme [40].

4. Identification of Selected Bacteria

4.1. Colony Morphology and Biochemical Tests

The effective three bacterial strains from the insect gut were isolated from nutrient agar was further subcultured on fresh sterile agar plates by a streak plate method and incubated 37°C for 24 hrs. According to the Bergey's manual, the selected three bacterial strains have been analyzed and its colony characteristic, biochemical properties were documented through visual and light microscopic observations [41, 42].

5. Molecular Identification

5.1. Genomic DNA Extraction of Bacteria

Genomic DNA extraction of bacteria was carried out by means of InstaGene™ Matrix Genomic DNA Isolation kit. As per the directions the kit DNA was extracted [43].

5.2. Amplification of PCR and Purification of Products

27F AGAGTTTGATCMTGGCTCAG 20 and 1492R TACGGYTACCTTGTTACGACTT 22 and universal primers gene fragment was used for PCR amplification. Purification of PCR was done by Montage PCR Clean up kit (Millipore). Sequencing reactions have been done with AmpliTaq® DNA polymerase (FS enzyme) (Applied Biosystems).

5.3. Sequencing and Phylogenetic Analysis

NCBI blast tool was applied for identification of sequence similarity [44]. Phylogenetic analysis was done with PhyML 3.0 aLRT program with HKY85 as a model substitution. Tree 198.3 program have been applied for tree rendering [45].

6. Results

6.1. Screening of Selected Bacterial Strains from the Gut of Insect

In our experiment 106 strains of bacteria have been screened in insect gut of *E.alacris alacris*. The results are presented in Figure 1.

A) Control; B, C, D) Isolated bacteria on nutrient agar plates

Figure 1. Isolation of bacterial strains from the gut of insect

6.2. Larvicidal Bioassay

All the bacterial metabolites (from 106 strains) exhibited larvicidal activity against *Cx. quinquefasciatus* subsequent to 24 hrs of the exposure phase. Among the secondary metabolites tested, an experiential larvicidal activity found in three bacterial strains. Based upon their LC₅₀ and LC₉₀ values the selected strains were S7, S8 and S37. Values of LC₅₀ of S7, S8 and S37 were 6.66ppm, 7.13ppm and 7.64ppm respectively. LC₉₀ values of S7, S8 and S37 were 115.90ppm, 205.73ppm and 242.59ppm respectively (Table 1).

Table 1. Larvicidal activity of *Eyprepocnemis alacris alacris* guts microbes against the larvae of *C. quinquefasciatus* (Say) at 24 hrs

Mosquito species	Microbial Culture	LC ₅₀	95% Confidence Limits		LC ₉₀	95% Confidence Limits	
			LCL	UCL		LCL	UCL
<i>Culex quinquefasciatus</i>	<i>Klebsiella pneumoniae</i> (S7)	6.66	0.43	19.46	115.90	70.27	170.07
	<i>Enterobacter xiangfangensis</i> (S8)	7.13	0.49	20.46	205.73	133.76	344.03
	<i>Ochrobactrum intermedium</i> (S37)	7.64	0.68	19.99	242.59	160.03	427.82

A: Control; B: S7; C: S8; D: S37; AG: Anal Gills; PM: Peritrophic Membrane

Figure 2. Peritrophic membrane and anal gills of dead larvae of *Cx. quinquefasciatus*

In the present investigation different larval symptoms were observed throughout the experimental period. In the initial stages of experimental study larvae have been vigorous and have typical movement. After that, each larva possessed a slight variation in their movement. After 24 hrs time interval the fatal larvae have been depicted under light microscope, where the body attained dark blackish colour, expatriation of peritrophic membrane and anal gills from the digestive system can be observed in the anal region and the internal part of the body and outer layer of skin were eroded (Figure 2).

6.3. Colony Morphology and Biochemical Tests

Bergey's manual of determinative bacteriology was used to analyse the genus level of each strains primarily. Gram staining of the three strains from insect gut using a light microscope showed that the three strains were Gram-negative and rod-shaped (Table 2). The analysed 3 strains were belonged to *Klebsiella*, *Enterobacter* and *Ochrobactrum* genera. The strain S7, S8 & S37 showed a

positive reaction on citrate utilization, urease, nitrate reduction, glucose, lactose and arabinose production and no reaction on H_2S production, lysine & ornithine utilization. S7 & S8 also showed a positive reaction on phenylalanine deamination, sorbitol and adonitol but S37 have no reaction phenylalanine deamination, sorbitol and adonitol production (Table 3).

6.4. Molecular Identification of Selected Gut Bacteria

Molecular identification of the three strains showed a high degree of similarities when contrasting the contig regions with NCBI GenBank entries using BLAST algorithm (<http://www.ncbi.nlm.nih.gov>) revealed to S7 strains have 93% maximum identity with a sequence of *Klebsiella pneumoniae*, S8 strains has 86% similarity with *Enterobacter xiangfangensis* and 78% similarity with (S37) *Ochrobactrum intermedium* (Figure 3, 4, 5). Neighbour-joining method was used to construct the phylogenetic tree.

Table 2. Colony morphology and physiology of isolated bacterial strains

Characteristic features	<i>Klebsiella pneumoniae</i> (S7)	<i>Enterobacter xiangfangensis</i> (S8)	<i>Ochrobactrum intermedium</i> (S37)
Gram stain	-	-	-
Morphology	R	R	R
Spores	-	-	-
Motility	-	+	-
Pigmentation	-	Cream	-

(R)- rode shaped, (+) – Positive, (-) - negative

Table 3. Biochemical analysis of selected bacteria

Tests	<i>Klebsiella pneumonia</i> (S7)	<i>Enterobacter xiangfangensis</i> (S8)	<i>Ochrobactrum intermedium</i> (S37)
Citrate Utilization	+	+	+
Lysine Utilization	-	-	-
Ornithine Utilization	-	-	-
Urease	+	+	+
Phenylalanine Deamination	+	+	-
Nitrate Reduction	+	+	+
H ₂ S Production	-	-	-
Glucose	+	+	+
Adonitol	+	+	-
Lactose	+	+	+
Arabinose	+	+	+
Sorbitol	+	+	-

(+: Positive Reaction, (-): Negative Reaction

Figure 3. Phylogenetic tree of *Klebsiella pneumoniae*.

Figure. 4. Phylogenetic tree of *Enterobacter xiangfangensis*

Figure 5. Phylogenetic tree of *Ochrobactrum intermedius*

7. Discussion

Resistance of mosquitoes to insecticides and chemical agents have been increasing rapidly. Searching of new molecules from natural environments is expected in the next decade. In our study suggested that the identified three insect gut microbes have insecticidal property on mosquito larvae. However, the derivatives of microbes could reduce the mosquito population and may alter the use of synthetic insecticides.

Efforts have been made to fight against *Anopheles gambiae* to control malaria and arbovirus infections and various concentrations had to influence the mortality percentage [46]. In this study revealed that *B. thuringiensis*, *S. aureus*, *M. sedentarius*, *E. faecalis* and *S. pneumonia* had larvicidal activity. In consonance with another [47] suggested that mode of action of bacterial species towards larvae could be due to the toxin production during their sporulation.

Xenorhabdus and *Photorhabdus* bacteria have possessed insecticidal activities against *Aedes aegypti* [48, 49]. Here, the fed group of *Xenorhabdus stockiae* had the highest larvicidal activity against *Aedes aegypti* (99% mortality) at 72 h after exposure and unfed group with 87% mortality at 96 h after exposure. *Xenorhabdus indica* had a high mortality rate against *Aedes albopictus* (82%) at 96h and unfed with 96% mortality rate at 96h. In accordance with our results exhibited that *Klebsiella pneumoniae*, *Enterobacter xiangfangensis* and *Ochrobactrum intermedium* had the highest larvicidal activity against *Culex quinquefasciatus* (100%) at 24h.

Experimentally, *B. thuringiensis* were effective in reducing the density of immature dengue vectors due to the self-spreading properties of its monolayers [50]. Formerly, it has been found *B. thuringiensis* and *B. sphaericus* are effective against mosquito larvae and safe to non- target organisms [51]. The findings of this study suggested the demonstration of LLMLFourstar® in natural mosquito larval habitats will not alter the vertebrates and invertebrates inhabiting with mosquito larvae in the aquatic environment.

Protein inclusions in the range of 60KDa- 150KDa existing in *B. sphaericus* can cause toxic effects to mosquito larvae [52]. In *Caenorhabditis elegans* cell death was triggered by the crystal toxins Cry6Aa- affecting the necrosis signalling pathway [53]. On the basis of this information, further studies are needed to identify the mechanism of action of three bacteria on *Culex quinquefasciatus* larvae to control mosquito community.

Klebsiella pneumoniae, *Enterobacter xiangfangensis* and *Ochrobactrum intermedium* have been isolated from the gut of *E. alacris alacris*, which have earlier been reported in the insect order of Dipterans and Lepidopterans [54]. *Ochrobacterium intermedium* also isolated from the gut of sand fly (Diptera: Psychodidae) and *Phlebotomus duboscqi* (Diptera: Psychodidae) [55, 56].

The three-insect gut microbial isolates, *Klebsiella pneumoniae*, *Enterobacter xiangfangensis* and *Ochrobactrum intermedium* caused persuasive mortality on the treated larvae. Likewise, species of *Enterococcus* and *Enterobacter agglomerans* isolated from *Melanoplus sanguinipus* showed insecticidal activity against agricultural pests. This result is in accordance with the metabolic action of *S. citreofluorescens* against mosquito larvae [57]. Exotoxins produced by the *Pseudomonas* species have been known to exhibit cytotoxicity against mosquito larvae [58].

8. Conclusions

To our knowledge, insecticidal activity of microbes screened from *Eyprepocnemis alacris alacris* gut was documented in the first time. Inference of this study suggested that enteric microbes in insects' gut could be used in order to produce bioactive compound against mosquito larvae.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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