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A NOVEL MOSQUIOCIDAL BACTERIUM FROM SOILS OF VELLORE, TAMIL NADU, INDIA

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ABSTRACT

In this study, intensive screening for mosquitocidal bacteria from different agricultural soils of Vellore district of Tamil Nadu was carried out to isolate potential mosquitocidal bacteria. From a total of 315 soil samples, 945 bacterial isolates were cultured, out of which three isolates showed mosquitocidal activity. One most potential mosquitocidal isolate was selected out of these and studied further. The isolate was identified by ilvD primer and phylogenetic tree as *Bacillus thuringiensis israelensis*. This new isolate exhibited effective toxicity against *Aed. aegypti, An. stephensi*, and *Cx. quinquefasciatus*. The LC_{50} values were 0.757, 0.52 and 0.165 mg/ l respectively. Similarly, the LC_{90} values were 0.963, 0.794 and 0.355 mg/ l, respectively. These results depict that the new isolate (*Bti*) VEVP-60 is a good candidate for mosquito control.

Key words: Red soil, *ilvD*, *Bacillus thuringiensis israelensis*, morphology, bioassays, mosquito larvae, *Aedes aegypti*, *Culex quinquefasciatus*, *Anopheles stephensi*, LC_{50}, LC_{90}, phylogeny.

Globally, mosquito vectors continue to have profound effects on the human and animal community as they transmit many life-threatening diseases. (Athni et al., 2021; Folly et al., 2020). Therefore, vector control plays a significant role in curtailing disease transmission (Wilson et al., 2020). The foremost and most effective intervention to control mosquitoes is to reduce their breeding in the immature stages as it is cost effective and provides long term control (Derua et al., 2019). Although chemical pesticides are effective in controlling these vectors, their persistent and repeated utilisation of chemical mosquitocides has created a global problem of resistance among the mosquito population (Meier et al., 2022). Additionally, they also affect the non-target species in the surroundings leading to ecological instability (Senthil-Nathan, 2020). All of these have led to find an alternative eco-friendly and effective control by employing micro-organisms (microbial control) (Hegazy et al., 2022). Many microorganisms have potential mosquitocidal properties, particularly bacteria (Silva et al., 2020). Hence in this study, with an aim to isolate highly potential and novel mosquitocidal bacteria, an attempt was made to explore different agricultural soil types in the Vellore District of Tamil Nadu, India.

MATERIALS AND METHODS

Soil samples weighing approximately 1-2 gm was collected and stored (4˚C) from various agricultural fields in the Vellore district (12.8937° N, 78.9741° E), Tamil Nadu, India. The samples are taken from three different levels of soil viz. at surface level, 5cm, and 10 cm depth separately. Later, in laboratory, the soil samples were serially diluted (10^{-4}) and spread uniformly in LB (Luria Bertani) agar plates for incubation at 30°C for 24 hr. After incubation, the appearance of various bacterial colonies was observed. Using a sterile loop, a single colony was inoculated in 10 ml LB broth. The inoculated culture is kept at 30°C in an incubator shaker (Scigenics Biotech LT4676, India) at 250 rpm for 72 hr. After 72 hr incubation, preliminary bioassay was carried out to screen for mosquitocidal bacteria. (Prasad et al., 2012). The bioassays were performed using late 3rd instar larvae of laboratory bred mosquito species (*An. stephensi, Cx. quinquefasciatus,* and *Ae. aegypti*) (WHO (2005), procured from the Mosquito Rearing and Colonization Unit of ICMR-VCRC (Manikandan et al., 2023). Doses of 1 and 10 µl of 72 hr bacterial culture were treated in each bioassay cup with appropriate controls and replicates. After 24 hr exposure, the larval mortality in each cup was recorded. If the larval

mortality in control is <5%, it was corrected using Abbot's formula (Abbott, 1925):

The extracted DNA of the most mosquitocidal isolate was amplified by polymerase chain reaction by using bacillus species-specific gene markers like *ilvD* under optimized primer and cycling conditions (Priest et al., 2004). The amplified PCR DNA was purified and cycle sequencing was performed by using PCR purification kit (QIAGEN, USA) and BigDye terminator V3.1 kit (Applied Biosystem), respectively. Further purification was done using the Macherey Nagel Nucleoseq purification column. Sequencing was carried out using the 3130XL Genetic Analyser facility (Applied Biosystem), Vector Control Research Centre, Pondicherry. The chromatogram was evaluated with Chromas (Version 2.01) and the consensus sequence was made using Bio-Edit (7.0.9.0). The identified consensus sequence was then analysed using the nucleotide blast program on NCBI. Identification of the isolate was done by constructing the phylogenetic tree using the Neighbour Joining model with 1000 bootstrapping in the MEGA programme (version 10.2.6) (Poopathi et al., 2014; Manikandan et al., 2023). The potential larvicidal isolate was further cultured and centrifuged at 10,000 rpm at 4˚C for 20 min in Hi-speed refrigerated centrifuge CR22GIII (Hitachi Koki Co. Ltd., Japan). Then the cell pellet was lyophilised and powdered. The resulting cell pellet powder was diluted in different concentrations to conduct toxicity bioassay (WHO, 2005). After, 24 hr treatment, mortality results were noted to determine its LC_{50} and LC_{90} by statistical probit analysis (SPSS 16.0, IBM Corp, USA). Colony morphology of the isolate was observed and compared to the WHO standard strain. Spore staining and gram staining was done for microscopic studies (Olympus CX41RF binocular microscope, Japan).

RESULTS AND DISCUSSION

In the present study, a total of 315 soil samples from various soil types in different agricultural fields and in varying depth were processed. From these 945 bacterial colonies were isolated and cultured. After 72 hr culture, preliminary bioassay screening against the larvae (late 3rd instar) of *Cx. quinquefasciatus, An. stephensi* and *Aed. aegypti* showed that three isolates showed toxicity. These isolates were coded as VEVP-60, VCRC-651, and VCRC-652. Further, the bacterial isolate was recultured and stored in glycerol for further analysis. Out of these three isolates, one most potential isolate *B. thuringiensis israelensis* (VEVP-60) isolated from the red soil of Velampattu village was selected and studied further. It can be observed that all three isolates were isolated from the top layer of the soil. Correspondingly, a study conducted in agricultural soils of Iowa (United States) concluded that there is decreased richness in microbial community with increasing depth (Hao et al., 2021). This can be attributed to the abundance of nutrients and organic matter in the surface level when compared to the subsurface layers (Seuradge et al., 2017).

For identification of the strain, the extracted DNA was amplified for *ilvD* (556bp) genes. The sequence was examined and the consensus sequence was obtained by Chromas and Bio-Edit. Through the Neighbour Joining model, phylogenetic tree was built with 1000 bootstrap replications in MEGA software. The isolate was identified as *B. thuringiensis israelensis* by *ilvD* markers. The phylogenetic tree shows, the consensus sequence of VEVP-60 has close similarity (100%) with *B. thuringiensis israelensis* (Fig. 1). Earlier study, reported that utilising *ilvD, pur*, and *pycA*, 47 *B. cereus* food-borne isolates was identified (Cardazzo et al., 2008). Manikandan and his coworkers reported that the molecular markers such as *ilvD, pur*, and *pycA* were used to identify the mosquitocidal bacteria *B. cereus* VCRC-B641 (Manikandan et al., 2023). However, whole genome sequencing of the DNA of *B. thuringiensis israelensis* will be useful as it is highly reliable in the identification of isolates.

After further processing, the lyophilized cell mass of *B.thuringensis israelensis* was subjected to detailed bioassays against the major mosquito vectors. The LC_{50} and LC_{90} (mg/ l) values of *Cx. quinquefasciatus* 0.17 and 0.35, for *An. stephensi 0.*52 and 0.79 and for *Aed. aegypti* 0.76 and 0.96, respectively (Table 1). *Cx. quinquefasciatus* is more susceptible, followed by *Anopheles stephensi* and *Aed. aegypti* (*Cx. quinquefasciatus* >*An. stephensi* >*Aed. aegypti*). The toxicity results proved that the indigenous *Bti* VEVP-60 was effective against *Cx. quinquefasciatus*, which can be used for the successful control of filarial vector. In a recent study, a novel *Bt* strain (TOD651) was found to kill *Aed. aegypti* (0.011 µg/ ml) and *C. quinquefasciatus* (0.023 µg/ ml) larvae (Alves et al., 2023). Similarly, in Indonesia, three strains of *Lysinibacillus sphaericus* were found to be effective against *Aed. aegypti.* With LC_{50} value for Bs9-2-3 is 1.75 x 104cell/ ml), Bs9-1-5 is 6.23 x 104 cell/ ml and Bs2-1-2 is 7.17 x 106 cell/ ml (Dewi et al., 2023). Since the majority of the larvicidal bacteria for mosquito vectors are spore-forming

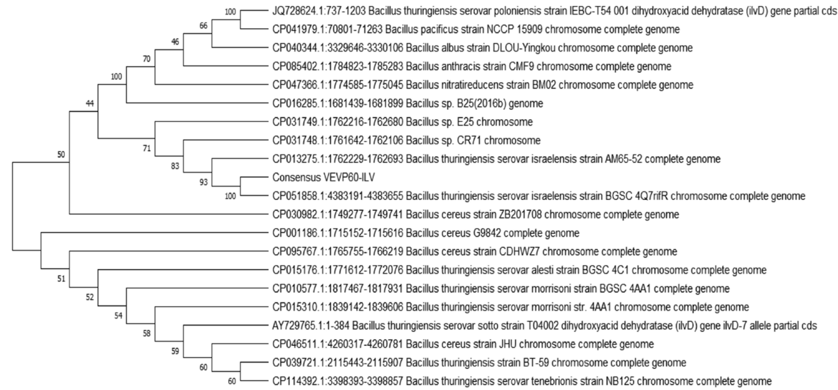

Fig. 1. Phylogenetic tree of *Bti – ilvD* gene using Neighbour joining model

Table 1. Mosquitocidal toxicity of the isolate of *B. thuringiensis israelensis* (VEVP-60)

Mosquito species	Slope	Intercept	*LC_{50} **(LCL- UCL)	*LC_{90} **(LCL- UCL)	χ^2 (df)
Aedes aegypti	0.003	-4.711	0.757 (0.703-0.813)	0.963 (0.912-1.056)	348.14
Anopheles stephensi	0.002	-2.425	0.520 (0.486-0.558)	0.794 (0.732-0.884)	46.81
Culex quinquefasciatus	0.003	-1.110	0.165 (0.104-0.221)	0.355 (0.287-0.484)	315.04

*mg/ l; **Lower confidential limit and upper confidential limit

bacteria, their spores can be preserved for a longer time, so these bacterial isolates can be cultured easily by supplementing them with appropriate nutrients and minerals. The colony morphology of the *Bti* VEVP-60 shows similarity with the international reference strain of *Bti*-H14 (IPS-82) i.e., white, almost circular with fine irregular margins (Ahmed et al., 2021). The colony morphology of the other two isolates show similarity to the reference strain *Bti*-H14; but it is to be noted that the three isolates showed varied potency across different mosquito vectors.

All the three isolates were gram stained and were identified as gram-positive. Generally, during soil processing, the diluted soil samples were subjected to heat treatment before plating, but in this experiment, it was not done to increase the chance of expecting mosquitocidal bacteria from both gram-positive and gram-negative groups (Geetha et al., 2014). Because most of the mosquitocidal bacteria reported are gram-positive, and only a few are gram-negative bacteria like *Pseudomonas fluorescens, Photorhabdus* spp., and *Xenorhabdus* spp. are known (Prabakaran et al., 2003; Subkrasae et al., 2022). After spore staining, it can be observed that in all isolates, the spores took green colour and crystals stained with pink colour, indicating that they are spore-forming, crystal-producing bacteria. Further analysis is needed to understand the intensity of their toxicity to mosquitoes and non-target organisms.

Several studies have emphasized the need for exploring biocontrol agents for safe and effective mosquito control. It is believed that the three isolates found from the farmlands of Vellore District of Tamil Nadu, India may prove important for upcoming production of bacterial pesticide for controlling mosquito vectors. Further, the mosquitocidal bacteria *Bti* VEVP-60 showed strong mosquitocidal activity against all three major mosquito vectors of *Cx. quinquefasciatus, An. stephensi* and *Aed. aegypti*. Therefore, it is concluded that the newly isolated bacterial strain of *B. thuringiensis israelensis* from red soil has its own impact on the control of mosquito vectors. Consequently, the strain of *Bti* can be used as an alternative to existing biopesticides, in the present scenario of resistance to *L. sphaericus*.

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AUTHORS CONTRIBUTION STATEMENT

PH was involved in the overall literature search, designing the study, sample collection, data collection, data interpretation and writing of manuscripts and critically revising the article. KA contributed by assisting in the statistical analysis of the experimental data. BB contributed by assisting in the field for soil sample collection, VA aided in the DNA extraction and data compilation. KG was involved in the molecular work. SM contributed to finding suitable journals, formatting references, checking for plagiarism, cleaning glassware, SM data interpretation, and data tabulation. JL contributed to the microscopic studies, and AM aided in interpreting the molecular studies and their plan. KV contributed to the initial review of the manuscript. SP was the PhD supervisor and contributed on the entire text of the MS.

CONFLICT OF INTEREST

No conflict of interest.

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