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Short Communication

Field evaluation of *Bacillus cereus* VCRC B540 for mosquitocidal activity – A new report

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Abstract. A major challenge to control the vector mosquitoes and their diseases. The discovery of bacteria like *Bacillus sphaericus* Neide (*Bs*) and *B. thuringiensis* serovar *israelensis* de Barjac (*Bti*), which are highly toxic to dipterans larvae, have opened up the possibility of their use as potential bio-larvicides in mosquito eradication programmes the world over. These bacteria have some important advantages over conventional insecticides in mosquito control operations, besides being safe for non-target organisms including human beings. But, the recent researchers have been reported mosquito resistant against these biological agents. *Bacillus cereus* VCRC B540 is one of the most potential bio-pesticides which were isolated from the gut contents of the marine fish (*Lutjanuas sanguineus*) collected in east coastal zone of the Bay of Bengal (Union Territory of Pondicherry, India) to control *Culex*, *Anopheles* and followed by *Aedes* species. The isolated strain was confirmed as *Bacillus cereus* based on the biochemical characteristics and 16S rDNA gene sequence. The larvicidal activity of *B. cereus* VCRC B540 was further characterized.

In recent years, there has been lot of improvements in the use of bio-pesticide as an effective tool for mosquito control. Several bio-control agents have been screened for their potency, mammalian safety and environmental impact since 20th century. There are several organisms investigated as potential agents for mosquito control, such as bacteria, virus, fungi, nematodes, protozoa, fish and invertebrate predators. However, the most of these agents have been shown to be of little operational use, largely because of the difficulty in multiplying them in large volume. Several spore forming bacteria and larvivorous fish can be undergoing extensive field trials. The discovery of bacteria like *Bacillus sphaericus* Neide (*Bs*) and *B. thuringiensis* serovar *israelensis* de Barjac (*Bti*), which are highly toxic to dipterans larvae, have opened up the possibility of their use as potential bio-larvicides in mosquito eradication programmes the world over. These bacteria have some important advantages over conventional insecticides in mosquito control operations, besides being safe for non-target species comprising human beings. Also it is harmless to the environment. In addition, these several other types of bacteria such as *B.t. jegathesan*, *B.t. morrisoni*, *B.t.* subsp. *malaysiensis*, *B.t.* subsp. *medellin*, *B.t.* subsp. *canadensis*, *Clostridium bifermentans*, *Asticcacaulis excentricus*, subsp. *malaysia* and *synechococcus* are being examined as

effective biological control agents. The *Bti* has been used for the control of mosquitoes for over two decades and its formulations are highly effective against *Anopheles*, *Aedes*, and *Culex* mosquitoes. There was no evidence was found that *Bs* and *Bti* toxins harm aquatic organisms in the breeding sites of these vectors or have an adverse effect on the environment. Although *Bti* is effective, specific, bio-degradable and have an extensive life, it does not recycle in the environment at points high enough to provide significant residual activity. It has a limited time of mode of action, usually 24 to 48 hours; hence, it applied at regular intervals. Besides, the current spore forming *Bti* formulations sink in water and are consequently less efficient in controlling of mosquito larvae that feed only close to the water surface. The killing effect on spores is slow compared with chemical insecticides and toxins have a narrower mosquito host range than the chemicals. *B. sphaericus*, on the other hand, has been shown to recycle in the field conditions and exert larvicidal activity for a long period. However, the spores of *Bti* have the benefit over *Bti* that *Bs* has a broader spectrum of activities against *Anopheles*, *Culex* and *Aedes* spp, while *Bs* has its effect mainly on *Culex* and to a lesser extent on *Anopheles*, and it is strongly species-specific and acts against only a few *Aedes* species. Resistance mechanism was reported against *Bs*, whereas resistance against *Bti* was more difficult due to the multiple toxin complex of this bacterium. With these knowledges, in the present study, the efficacy of the newly isolated bacterial strain of *B. cereus* VCRC B540 was field tested for the mosquito control activity against disease causing vectors.

Serial dilution was made from bacteria (*B. cereus* VCRC B540) isolated from marine fish in east coastal zone of the Bay of Bengal in the Union Territory of Puducherry (India) (Sambrook, 1989). The bacterial samples were serially diluted (10^{-6}) and plated on Luria–Bertani (LB) agar plates. Plates were incubated at 37°C overnight. The individual bacterial colony after confirmation of morphological features was selected and thereafter cultured and sub-cultured in 5 ml and 150 ml LB media (peptone, yeast extract, and NaCl 2:1:2, pH 7.8), respectively. The bacterial cultures have grown under stable agitation in an orbital shaker (200 RPM at 30°C for 72 h). The cell mass was harvested by centrifugation (10,000g/30 min/4°C) by SORVALL Evolution RC super-speed centrifuge (Kendro, USA), and pellets were lyophilized.

Liquid formulation from *B. cereus* VCRC B540 was made by mixing this bacterium (5%) with ingredients like citric acid, glycerol, liquid paraffin, Congo red, sodium benzoate, and sodium alginate (0.1%, 20%, 10%, 0.025%, 0.2% and 4%) respectively. The formulated product of *B. cereus* (Figure not shown) was stored at room temperature (30°C) until further use.

The liquid formulation was evaluated against three mosquito species of laboratory-reared early third instars (*Cx. quinquefasciatus*, *An. stephensi* and *Ae. aegypti*). A homogeneous stock solution of the liquid formulation was prepared and toxicity assay was carried out in disposable wax coated paper cups (350 ml capacity) under laboratory conditions at room temperature (28 ± 2). Serial dilutions were prepared by adding appropriate volumes of formulation in 100 ml of chlorine-free tap water containing 25 early third instar larvae of the respective mosquito species, separately. Triplicates of seven concentrations of tenfold of LC_{50} values (0.376, 0.188, 0.094, 0.047, 0.023, 0.011, 0.005 mg/l) leading to mortality rates between 1% and 100% were tested, and mortality was recorded after 24 hours (WHO, 1985). The experiments were carry out on different occasions with three duplicates in each experiment. Food supplement (biscuit and yeast mixture, 2:1) was provided for the larvae under treatment with bacterial toxin. Control mortality (if any) was corrected (Abbott, 1925).

Moribund larvae in the replicates were counted as dead. The data from bioassays were subjected to student't' test to analyze significance of difference of ($P \geq 0.05$). The LC_{50} and LC_{90} values were calculated from probit analysis using software package "ASSAYS".

The mosquito larvae breeding sites were selected for the field evaluation (Krishna Nagar, Thiru Nagar (Moolakkulam), Ellai Pillaisavadi, and Ninobabha Nagar), which was situated on the urban and semi urban area of Puducherry, South India. It was reported earlier that Moolakkulam was an endemic area for filariasis. The study area contained numerous drainages, ditches and cesspits, often fed by a wide sewage canal deriving from a neighbouring urban area, which permanently hold up heavy breeding by *Cx. quinquefasciatus*. The average surface area of experimental study site was 2–5 m^2, with average water depth between 1–2 m and pH from 7.6–8.5. The temperature ranged from 25ºC to 30ºC during the study period (July to December, 2014).

The *B. cereus* VCRC B540 based liquid formulation was field tested against the filarial vector (*Cx. quinquefasciatus*) in an urban area of Puducherry. Three replicates and one control cesspits of each site (Krishna Nagar, Thiru Nagar (Moolakkulam), Ellai Pillaisavadi, and Ninobabha Nagar) with high larval density were selected. The liquid formulation produced from bacterial toxin, along with other ingredients, was sprayed on the respective cesspits with hand-compression sprayer (Vol 2.5 l) at the rate of 0.25g/m^2 as described earlier (Poopathi *et al*., 2003). Pre and post-treatment counts of immature of *Cx. quinquefasciatus* in all habitats were done by taking three dips per cesspit using standard 350 ml dipper and the data were recorded. Three replicates were placed for test and control experiments. The larval samples were brought to the laboratory for identification. Post-treatment counts of larvae were carried out on alternative days until the immature population reached approximately pre-treatment levels. The percentage reduction (% R) immature was calculated by the Mulla's formula (Mulla *et al*., 1982).

$$(\%\ R) = 100 - \frac{(C1 \times T2)}{(T1 \times C2)} \times 100$$

Where C_1 is the number of larvae in control cesspits before treatment, C_2 is the number of larvae in control cesspits after treatment, T1 is the number of larvae in treated cesspits before treatment and T_2 is the number of larvae in treated cesspits after treatment.

Data pertaining to the laboratory and field evaluation with *B. cereus* VCRC B540 based liquid formulation were subjected to student's t test to analyze the significance of the difference. The LC_{50} and LC_{90} values were calculated by Probit analysis.

The bacterial strain was isolated as new by 16S rDNA sequences and their respective 16S rDNA sequences had been submitted to NCBI under the Acc. No JN377787. The liquid formulation was developed and tested in the laboratory against different species of mosquito (*Cx. quinquefasciatus*, *An. stephensi* and *Ae. aegypti*) with three replications at room temperature. The respective LC_{50} and LC_{90} values against mosquito larvae of *Cx. quinquefasciatus* were 0.047 g/m^2 and 0.37 g/m^2, respectively. The respective LC_{50} and LC_{90} values against mosquito larvae of *An. stephensi* were 0.068 g/m^2 and 0.4 g/m^2 respectively.

The potency of *B. cereus* VCRC B540 produced from Luria-Bertani medium was evaluated in the field for the control of the filarial vector of *Cx. quinquefasciatus*. The larval density of the experimental area was brought down by 80–100% within 24 h post-treatment. This considerable reduction of larval density in the cess pits continued for nearly three weeks (17 days). Later on, the larval density started increasing from the 19th day onwards (Fig. 1). The mosquito larvae killed by *B. cereus* toxin-based formulation were easily identified due to a dark coloration of dead larvae. These observations, demonstrated that there was a significant reduction in the sites due to treatments (two factors), period of exposure and their interaction where *B. cereus* VCRC B540 toxin based formulation used.

In the present study, the possibility of using *B. cereus* VCRC B540 based liquid formulation as a bio-pesticide in field application. However, this product has not been studied for the production of a bio-pesticide, like *Bti* in mosquito vector control programme, and this has been the objective of the present study. *B. cereus* VCRC B540

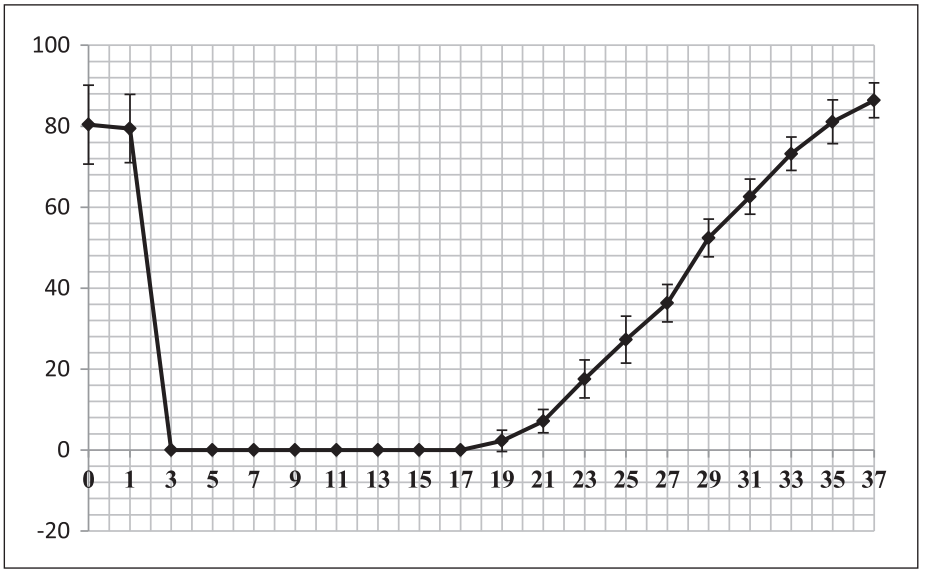

Figure 1. The larval control pattern in field after application of *B. cereus* VCRC B540 based formulation.
X axis : Days.
Y Axis : Reduction (%).

based liquid formulation is a potential candidate for mosquito control programme, since various reports of resistance to different strains and formulations of *B. sphaericus* in *Cx. quinquefasciatus* from different countries are being reported and it has been suggested that continuous exposure to *B. sphaericus* would result in the development of moderate to high level of resistance in *Cx. quinquefasciatus* (Poopathi *et al.*, 2014). Development of resistance to *B. sphaericus* has also been demonstrated in *An. stephensi* under laboratory selection.

In the laboratory bioassays, concentrations of 0.0047 mg/l and 0.037 mg/l resulted in 50% and 95% mortalities respectively after 24 hours of exposure to *B. cereus* VCRC B540 and in field the required concentration was 0.047 g/m^2. The target mosquito larvae tested (larvae of *Cx. quinquefasciatus*) was extremely sensitive to the *B. cereus* VCRC B540 liquid formulation (Krishna Nagar, Thiru Nagar (Moolakkulam), Ellai Pillaisavadi, and Ninobabha Nagar), with the most sensitive stage being the early instars. This study confirms 17 days residual activity of *B. cereus* VCRC B540 like *B. sphaericus*, which is better than residual activity of *Bti* demonstrated earlier (Margalit & Dean, 1985). *Penaeus monodon* (Prawn), *Oriachromes mosambicus* (Tilapia), *Cantareus aspersus* (common snail), *Scylla serrata* (mud crabs), *Daphnia lumholtz* (Daphnia), *Gambusia affinis* (fish), *Antipodophlebia asthenes* (Dragon fly nymph) were used for non-target analysis which was collected from where the non-target organism associated with mosquito larvae. The liquid formulation did not show any activity against other non-target organisms (Table 1).

Thus, present results indicate that *B. cereus* VCRC B540 based liquid formulation can be considered as a replacement to synthetic chemicals. The present study also corroborates the earlier reports that *B. cereus* as potential bacterium for mosquito control. In earlier, many researchers have been reported *B. thuringiensis* subsp. *israelensis* (*Bti*) to be an effective larvicide. However, literature indicates that an effective evaluation and comparison of *Bti* -based formulations when tested in streams or

Table 1. Effect on non-target organisms by using formulated *B. cereus* VCRC B 540

Non target organisms	Exposures (hrs)							
	24 (Hrs)			Average	48 (Hrs)			Average
	R1	R2	R3		R1	R2	R3	
Penaeus monodon (Prawn)	–	–	–	–	–	–	–	–
Oriachromes mosambicus (Tilapia)	–	–	–	–	–	–	–	–
Cantareus aspersus (Common snail)	–	–	–	–	–	–	–	–
Scylla serrata (Mud crabs)	–	–	–	–	–	–	–	–
Daphnia lumholtzi (Daphnia)	–	–	–	–	–	–	–	–
Gambusia affinis (Fish)	–	–	–	–	–	–	–	–
Antipodophlebia asthenes (Dragon fly nymph)	–	–	–	–	–	–	–	–

rivers is difficult. Most field trials have been conducted in different rivers (different discharge, river profile, water temperature, suspended matter, larval species, etc.), thus rendering the evaluation of the performance of liquid formulations of *Bti* very arbitrary or even impossible. Aqueous suspension or flowable liquid formulation generally produced better results against column feeding *Culex* mosquitoes, while surface spreading formulations or dust formulations were more effective against surface feeding *Anopheles* species and granular and tablet formulations were more effective against *Ae. aegypti* (Poopathi *et al*., 2002).

The efficacy of bacterial preparations against target mosquitoes is influenced by various physico-chemical and biotic factors such as temperature, water pH, sunlight, sedimentation rate of spores, organic pollution, larval stage, density, etc. Temperature is a significant factor, which influences the toxicity of these bacterial preparations and also pH of the water has been found to influence the activity of bacterial preparations. *B. cereus* VCRC B540 is highly effective against *Culex* species, even in highly polluted waters and also has longer impact on larval populations. However, development of genetically engineered recombinant strains by cloning of toxin genes (SLP) of *B. cereus* VCRC B540 might help in broader spectrum of activity and in delaying the development of resistance.

It was observed that *B. cereus* VCRC B540, as evaluated in the field trial, was effective in controlling the filarial vector of *Cx. quinquefasciatus* (0.047 g/m^2). Therefore it is concluded that *B. cereus* VCRC B540 based formulation has the potential for field application to control mosquito vectors causing lymphatic filariasis.

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Conflict of Interest

All authors report no conflicts of interest relevant to this article.

REFERENCES

Abbott, W.S. (1925). A method of computing the effectiveness of an insecticide. *Journal of Economic Entomology* **18**: 265-267.

Margalit, J. & Dean, D. (1985). The story of *Bacillus thuringiensis* var. *israelensis* (*Bti*). *Journal of the American Mosquito Control Association* **1**: 1-7.

Mulla, M.S., Federici, B.A. & Darwazeh, H.A. (1982). Larvicidal efficacy of *Bt* serotype H-14 against stagnant water mosquitoes and its effects on non-target organisms. *Environmental Entomology* **11**: 788-795.

Poopathi, S., Anupkumar, K., Kabilan, L. & Sekar, V. (2002). Development of low-cost media for the culture of mosquito larvicides, *Bacillus sphaericus* and *Bacillus thuringiensis* serovar *israelensis*. *World Journal of Microbiology and Biotechnology* **18**: 209-216.

Poopathi, S., Anupkumar, K., Arunachalam, N., Tyagi, B.K. & Sekar, V. (2003). A small scale mosquito control field trial with the biopesticides *Bacillus sphaericus* and *Bacillus thuringiensis* serovar *israelensis* produced from a new culture medium. *Biocontrol Science and Technology* **13**: 743-748.

Poopathi, S., Mani, C., Vignesh, V., Lakshmi Praba, V. & Thirugnanasambantham, K. (2014). Genotypic diversity of mosquitocidal bacteria (*Bacillus sphaericus*, *B. thuringiensis*, and *B. cereus*) newly isolated from natural sources. *Applied Biochemistry and Biotechnology* **171**: 2233-2246.

Sambrook, J., Fritschi, E.F. & Maniatis, T. (1989). Molecular cloning: a laboratory manual, Cold Spring Harbor Laboratory Press New York.

WHO (1985). Informal consultation on the development of *Bacillus sphaericus* as a microbial larvicide. *TDR/BVC/sphaericus/853/WHO/VBC* 1–24.