Efficacy of Native *Bacillus thuringiensis* against Mosquito Vector

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ABSTRACT

Background: Larval source management is an effective measure to control mosquito-borne diseases. Bacillus thuringiensis produces specific insecticidal crystal proteins toxic to mosquito larvae. In many parts of the South East Asian region, Bacillus thuringiensis is used for larval source management. In Nepal, larvicidal Bacillus thuringiensis is not available. The study aims to isolate larvicidal Bacillus thuringiensis from soil samples of Nepal to control mosquitoes.

Methods: Native Bacillus thuringiensis was obtained from soil samples by the acetate selection method. It was identified by observing crystal protein with Coomassie Brilliant Blue stain in a light microscope. The mosquito larvae were collected from different breeding habitats. A preliminary bioassay was performed by inoculating three loopful of 48 hours culture of spherical crystal protein producing Bacillus thuringiensis in a plastic cup containing 25 larvae and 100 ml of sterile distilled water. The cup was incubated at room temperature for 24 hours to observe the mortality of larvae. Further selective bioassay was performed with the isolate which showed 100% mortality, as described above in four replicates along with the negative and positive control.

Results: Out of 1385 Bacillus thuringiensis obtained from 454 soil samples, 766 (55.30%) were spherical crystal protein producers, among them, a single strain (14P2A) showed 100% mortality against mosquito larvae. The lethal concentration doses required to kill 50% and 90% of the larval population were 32.35 and 46.77 Parts per million respectively.

Conclusions: The native Bacillus thuringiensis produces the crystal protein effective in killing mosquito larvae. The native Bacillus thuringiensis should be included as a tool to control mosquito-borne diseases in Nepal.

Keywords: *Bacillus thuringiensis*; mosquito; Nepal; tool.

INTRODUCTION

Mosquito-borne diseases (MBDs) have expanded from endemic regions to nonendemic regions due to climate change, urbanization, globalization, changes in mosquito behavior, etc. [1-4] Newer interventions are required to address the emerging situation of diseases. In recent years, larval source management (LSM) has been focused as a supplementary approach to manage larval habitats to prevent the development of adult mosquitoes. [5-7]

Bacillus thuringiensis subspp *israelensis* is recommended by WHO for LSM. [8] Nepal lacks *Bacillus thuringiensis* (Bt) and other biological control agents. No attention is paid to these types of vector control interventions in Nepal. For sustainable vector control, it is essential to focus on larval source reduction by using Bt which is a safer, cost-effective, and environmentally acceptable tool.

The research aimed to isolate and identify Bt from the soil samples of Nepal, and to screen the isolates for larvicidal activity against field collected mosquito larvae

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by preliminary and selective bioassay and comparing its toxicity with the standard strain.

METHODS

This study was carried out in the laboratory of Nepal from 2020 to 2022. The research target was to isolate mosquito larvicidal *Bacillus thuringiensis* (Bt) from the soil samples of Nepal. To test the larvicidal activity of the isolated Bt, mosquito larvae were collected from the Kathmandu and Lalitpur districts of Nepal from different types of breeding habitats. Ethical approval was granted by Nepal Health Research Council to carry out the research work (Regd.No. 79/2020).

Soil sample (10 gram) was collected randomly from the seven Provinces of Nepal in a Ziplock bags, five centimetre below the surface by clearing the decaying leaves and other organic matter. [9] Soil samples were collected from a diverse location as possible, forest, fertile land, uncultivated land, nearby ponds, lakes, stream, university premises, parks, etc. [10] Collected soil sample was brought to the laboratory and stored at room temperature before isolation of native Bt.

Isolation of *Bacillus thuringiensis* (Bt) was carried out by the acetate selection method, one gram soil was added to a 10 ml test tube containing nine millilitres of nutrient broth (NB) supplemented with 0.25M sodium acetate. It was incubated overnight at 28^{0}C in an incubator. After enrichment, the broth was exposed to heat shock (100^{0}C) in a water bath for five minutes with slight modification. [11] A spread plate technique was performed for the isolation of Bt in nutrient agar (NA) by loading 100µl of serially diluted nutrient broth in a saline solution after heat shock. The plates were incubated at 28^{0}C for 48 hours in an incubator. The isolated colonies were further subcultured to obtain the pure culture in NA.

The isolates were identified as Bt from a 48 hours culture plate by observing Coomassie brilliant blue stained (0.133% Coomassie Brilliant Blue (CBB) G250 in 50%acetic acid) crystal protein and its shape in light microscope. [12] Biotyping like Gram staining, spore staining, catalase, oxidase, motility test, starch hydrolysis, gelatin hydrolysis, casein hydrolysis, and Tween 20 hydrolysis were performed and the results were recorded after 48 hours. Isolates identified as Bt were preserved in a nutrient agar slant with 60% glycerol.

Mosquito larvae were collected from different breeding habitats in Kathmandu and Lalitpur districts during 10 months period (June - September 2021 and April - September 2022) using dropper and dipper. [13-15] The collected larvae were transferred into a transparent plastic bag with water and brought to the laboratory. Thereafter placed in a tray containing distilled water for two days for acclimatization before using them for larvicidal activity. Meanwhile, larvae were fed with the baker's yeast and dog biscuits. [16] The larvae preceded to pupae were collected and removed from the tray.

For preliminary screening, 25 larvae, size range (9mm -14mm) equivalent to third instar to early fourth instar were placed in a plastic cup containing 100 ml sterile distilled water. [17] Only the acclimatized healthy and active movement showing larvae were used for the larvicidal activity. Fresh culture of Bt isolates in a nutrient agar plate after 48 hours of incubation was taken for the assay. With the help of a standard, sterilized loop, three loopful of culture were scooped (≈ 0.03milligram or 300 Parts per million (ppm)) and added to the cups containing larvae, homogenized gently without harming them. The culture contained a crude mixture of spores, crystal proteins, and vegetative cells. [10,18] The cups were incubated at room temperature for 24 hours the experiment was performed in August and September 2021 and 2022. The average room temperature during the analysis was 24.5^{0}C and a humidity of 66% was recorded from the mobile app (Room Temperature Thermometer, Morsol Technology). After 24 hours the numbers of dead, morbid, and live larvae were counted. The experiment was repeated when more than 10% of pupae were pupated from the larvae within 24 hours. The Bt isolate showing larvicidal activity was further confirmed by selective bioassay. [10]

Standard reference strain *Bacillus thuringiensis* var *israelensis* (Bti) IPS-82 was purchased from Pasteur Institut, Paris, France. The control strain was cultured in the laboratory condition in NA. The phenotyping and biotyping of the standard strain were performed along with the native strain in the same environmental condition. The larvicidal activity of the control strain was performed along with the native Bt isolates.

A selective bioassay was performed after the preliminary screening of Bt showing 100% mortality against the field collected larvae. Selective bioassay was performed in four replicates. In a plastic cup, 100 ml of sterile distilled water and 25 larvae of third instar to early fourth instar larvae were added to each cup. Fresh culture of native Bt showing 100% mortality during the preliminary screening was added by using a standard and sterilized loop, as described for the preliminary

screening. Similarly, for positive control fresh culture of standard strain Bti IPS-82 was added to four cups as of the test Bt. Negative control cups contain 25 larvae and 100 ml of sterile distilled water in every four cups. All the cups were placed at room temperature for 24 hours. After 24 hours the numbers of live larvae were counted. [17] The experiment was repeated if more than 10% of pupae were pupated from the larvae in 24 hours period in negative control or positive control and also in the test cups. The mortality percentage was calculated by using the formula

Mortality (%) = ((X -Y)/X) x100

Where X = percentage survival in the untreated control and Y = percentage survival in the treated sample. [17]

To confirm that the larval death was due to feeding of the toxin of the inoculated Bt or other contaminations. The single dead larvae were removed from the positive control cup and test cup, washed with sterile distilled water, and surface sterilized with 70% ethanol twice after evaporation of the alcohol the larva was crushed in a test tube with a sterilized glass rod in a one millilitre of sterile saline solution. Then 100 µl of the saline solution was spread with a sterilized "L" shaped glass rod on the nutrient agar surface and incubated at 28^{0}C for 48 hours.

For confirmation test that all spherical crystal proteins producing Bt is not toxic to the mosquito larvae was done by inoculating 14P2A and 3P2A (spherical ICPs producing Bt) 48 hours fresh culture colony in a cup containing 25 larvae and 100 ml sterile distilled water as mentioned elsewhere.

Lethal concentration doses required to kill 50% and 90% of the larval population was determined by culturing 14P2A in NB broth incubated for 72 hours in a shaker water bath at 28^{0}C. One percent working solution was prepared from the broth in a sterile distilled water. Following microliter (1000, 500, 400, 300, 200, and100) of the working solution was added to the cups containing 25 larvae in four replicates as described elsewhere and by using probit analysis the LC_{50} and LC_{90} value was determined.

Results were presented in tables, and figures where applicable. Bioassay was performed in four replicates and the mean mortality percentage was calculated as described by WHO [17] manually. LC_{50} and LC_{90} was determined by using Probit analysis. The mortality due to the production of crystal protein was confirmed by comparing it with the standard strain Bti-IPS-82 and the untreated control group. All the experiments were carried out in a room temperature with a photoperiod of 14:10 (L: D) at an average temperature of 24.5^{0}C and 66% relative humidity.

RESULTS

Altogether 1385 Bt isolates were obtained from 454 soil samples collected from different ecosystems such as forests, uncultivated land, university area, etc covering seven provinces of Nepal. On average each soil sample contains greater than three morphologically different types of Bt isolates. The isolates which produced additional structure (crystal protein) apart from the spore and vegetative cell, observed by CBB stain in a light microscope were identified as Bt isolates. The absence of the additional structure in the isolates was assumed as other types of *Bacillus* and not included in the study.

The native Bt isolates produced different shapes of crystal protein (Table 1). The dominant Insecticidal crystal proteins (ICPs) were spherically shaped.

Table 1. Shapes of Insecticidal Crystal Proteins (ICPs).

Shapes	No. of Bt
Spherical	766 (55.30%)
Bipyramid	3 (0.21%)
Rod shaped	49 (3.53%)
Cap headed	502(36.24%)
Spherical + cap headed	42(3.03%)
Oval	23(1.66%)
Total	1385

On preliminary screening of 808 Insecticidal Crystal Proteins (ICPs) producing Bt isolates for larvicidal activity (spherical (766) and spherical + cap headed (42)), only one isolate coded as 14P2A, producing spherical ICPs showed 100% mortality against mosquito larvae (Figure 1). The native larvicidal Bt 14P2A was among the 766 Bt isolates producing spherical shaped ICPs. The Bt isolates producing both spherical and cap headed (42) and the 765 spherical ICPs producing native Bt isolates lack the mosquito larvicidal property.

Selective bioassay proved the isolate's ability to show 100% mortality as the standard strain within 24 hours at 300 ppm (Parts per million) concentration (Figure 1 and Table 2). So, the selective bioassay confirms that 14P2A is a mosquito larvicidal *Bacillus thuringiensis*. Further

confirmation with 3P2A also proved 14P2A as mosquito larvicide Bt were as 3P2A lack the ability even though it possesses a spherical shaped crystal protein.

Table 2. Larvicidal activity of native Bt and the reference strain Bti (IPS-82) against field collected larvae.

Isolates	Conc. PPM	Total larvae	Live larvae	Dead larvae	Pupa	Adult	Mortality %
Bti (IPS-82)	300PPM	25(100) *	0	25(100)	0	0	100
Bt(14P2A)	300PPM	25(100) *	0	25(100)	0	0	100
3P2A	300PPM	25(100) *	25(100)	0	0	0	0
Control	-	25(100) *	25(100)	0	0	0	0

*Four replicates (each cup contains 25 larvae, four cup 100 larvae).

On analysis of dead larvae after 24 hours by spread plate technique it was found the same colony characteristic producing Bt were present in the dead larva. Therefore, the death of the larvae is by feeding on the crystal protein of the14P2A Bt isolate.

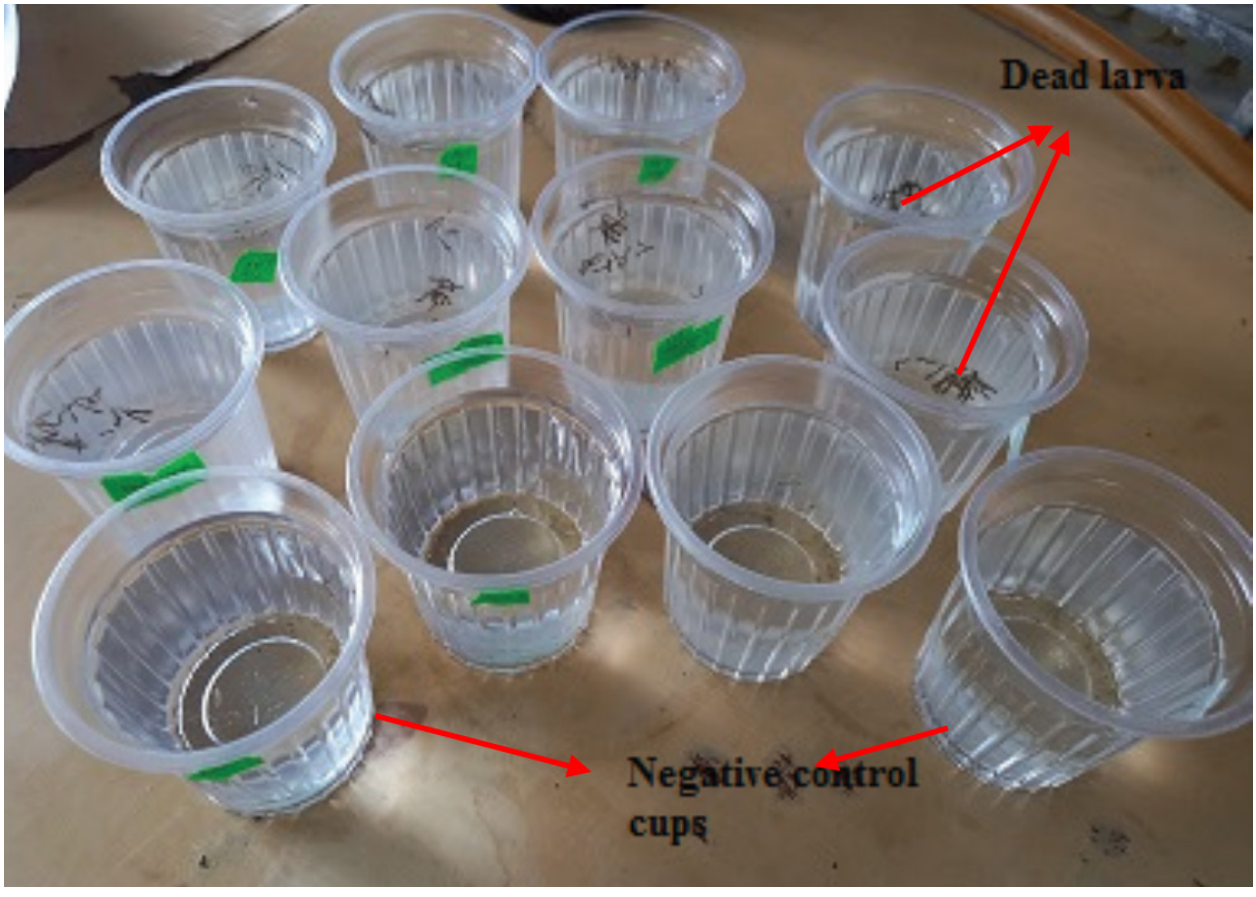

Figure 1.Selective bioassay. Four replicates of control strain, native strain and negative control.

The lethal concentration dose required to kill 50% and 90% of the larval population was found to be LC_{50} 32.35ppm and LC_{90} 46.77ppm against the field collected mosquito larvae. The colony morphology of the larvicide 14P2A in NA appears irregular in shape, and size (15mm- 30mm), dry, flat, and pale white (Figure 2D). The colony character of 14P2A is different from the colony characters of other spherical crystal protein producing Bt isolates. On biotyping it is Gram positive straight rod arranged singly (Figure 2A), on spore staining by Schaeffer Fulton stain, the spores were

elliptical in shape and the green spore in the vegetative cell is in sub terminal position without bulging (Figure 2B). On CBB stain the crystal protein appeared as blue spherical like in shape (Figure 2C) the elliptical spore was faint blue in color and the vegetative cells were dark blue in color.

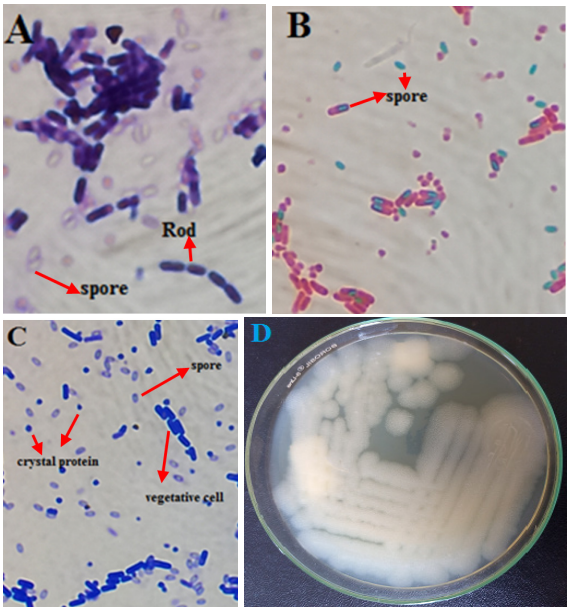

Figure 2.A.Gram staining, purple rod shaped bacteria and unstained elliptical spores. B.Spore staining, green elliptical spores, Red vegetative cells, inside the red vegetative cell the green spore is present at the sub terminal end of the vegetative cell and C.CBB staining of the isolate 14P2A, the crystal protein is dark somewhat spherical in shape. D. Culture plate of 14P2A in NA.

The control strain and the 14P2A were motile. The biochemical properties were catalase and oxidase positive as well as both of the isolates were able to hydrolyze different substrates like starch, gelatin, casein, and Tween20.

DISCUSSION

Soil samples are rich resources of Bt, we detected 1385 Bt strains from 454 soil samples. The result is in agreement with the result of Martin and Travers. [9] This research result also indicates that Nepal soil is rich in Bt biodiversity as different shapes of crystal protein producing Bt has been found. More than three morphologically different Bt varieties were obtained from a gram of soil sample. All the Bt isolates showed variation in colony morphology, fried egg type with excess mucous, and irregular shape were dominant in soil. All Bt isolates were Gram positive, spore bearing, but showed variation in the location of spores in a vegetative cell, in the shape of the spore, in cell size, and in the shape of the crystal protein as observed by light microscope with different staining techniques. [10,19,20] With the assumption of spherical crystal morphology of Bt, its specificity against the mosquito larvae. [8] Bt isolates producing spherical shaped crystal proteins of different sizes were screened for larvicidal activity. The spherical shaped crystal protein producing Bt were more dominant than the other types of crystal protein producer. [10,19,20] But the distribution of mosquito larvicidal Bt in Nepal was not found to be ubiquitous as only one (0.12%) mosquito larvicidal Bt coded as 14P2A was isolated from 454 soil samples. Relatively few strains of Bt with larvicidal activity against insects of public health importance have been discovered. Soares-da-silva et al 2015, [21] isolated five larvicidal Bt against *Ae. aegypti* out of 57 Bt. According to El-Kersh et al 2016, [10] 23 native Bt isolates were identified as larvicidal in the preliminary test against *An. gambiae*. Selective bioassay results also proved that the isolate 14P2A produces the crystal protein toxic to the mosquito larvae within 24 hours. Approximately 300 ppm of the spore, crystal, and vegetative cell mixture is enabled to show 100% mortality of larvae in the laboratory condition. The 100 larvae presented in the negative control cup were all found alive even in the absence of larval food and sustained in the larval stage. The field collected larvae were a mixture of different species of mosquito larvae. Mortality (100%) of field collected larvae showed its effectiveness (14P2A) in killing all kinds of mosquito larvae. Similarly, the control strain IPS-82 also showed 100% mortality against the field collected larvae.

Larvicidal activity evaluated in the present study may be due to the production of crystal protein which was more specific and not all types of spherical shaped crystal protein contain toxicity against the mosquito larvae. Various studies showed that the mosquito larvicidal Bt will kill all kinds of mosquito larvae and are also toxic to midges. [22-24] Therefore native larvicidal Bt 14P2A can be effective against larvae of *Aedes*, *Anopheles*, and *Culex* furthering, its efficiency as an environmentally friendly microbial control options of mosquito-borne diseases. Likewise, the larvicidal activity of 14P2A with 3P2A spherical crystal producing Bt with different colony morphology confirmed that the crystal protein composition was different in both of the isolates even though the shape of the ICPs was the same. ICPs of 14P2A are toxic whereas 3P2A ICPs are not toxic after 24 hours, the cup inoculated with 14P2A showed mortality of larvae as the cup with 3P2A contains alive larvae as in

negative control. On analysis of dead larvae by spread plate method in nutrient agar plate showed the organism present inside it was 14P2A. Because of nontoxic nature of Bt to humans. LSM by using Bt outdoor and indoor at regular intervals, in the water storing containers will not be hazardous. Furthermore, *Aedes* species are adapted to breed indoors. LSM by Bt can be an effective control measure, when by applying it into water holding containers for drinking, recreational and other purposes. [25] Considering the growth of 14P2A in NB broth as a bio larvicide, from the one percentage working solution of the bio larvicide, the lethal concentration doses required to kill 50% and 90% of the larval population was LC_{50} 32.35 ppm and LC_{90} 46.77 ppm against the field collected mosquito larvae.

Through the exploration of different samples and unique habitat such as dead insects, water, organic waste, phyllosphere, rhizosphere etc, more mosquito larvicidal Bt can be obtained which not only help to isolate a greater number of Bt but also their different native varieties leading to adopt more possibilities for prevention and control of mosquito-borne diseases in Nepal. In addition, the isolation of larvicidal activity led to further assay against laboratory reared different species of larval mosquitoes and to evaluate its efficacy in natural habitats as well.

CONCLUSIONS

Native *Bacillus thuringiensis* has been found to be effective in killing mosquito larvae. Nepal currently lacks biological control agents as part of its vector control program. Incorporating native Bt into the vector control program in Nepal could be a potential option for reducing the mosquito population and limiting the spread of mosquito-borne diseases. However, it's important to conduct further studies to evaluate the safety and effectiveness of using Bt in this context, including its impact on non-target species and the potential development of resistance in mosquito populations over time.

Overall, the use of native Bt as a biological control agent could be a useful addition to the toolbox for controlling mosquito-borne diseases in Nepal, but more research and evaluation is needed before its widespread implementation in vector control programs.

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