Soil Bacteria that Kill Mosquito Larvae

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ABSTRACT

Background

To control the deadliest mosquitoes, *Bacillus thuringiensis* var *israelensis* and *Bacillus sphaericus* are the tools to prevent the development of adult mosquitoes by killing the larvae in different water sources. Various countries formulated *Bacillus thuringiensis* and *Bacillus sphaericus* larvicide in different forms isolated from various sources to control mosquitoes. These bacteria are nontoxic for humans and other living organisms. In Nepal, unawareness and unavailability of these isolates made us inaccessible to these tools to control mosquito vectors. To address the increasing trend of mosquito-borne diseases in Nepal these bacteria should be included as a vector control tool.

Objective

To isolate and test the novel bacteria *Bacillus thuringiensis* and *Bacillus sphaericus* from the soil sample of Nepal as a biological mosquito control tool.

Method

Soil samples collected from seven provinces of Nepal were processed by two different techniques to isolate these two bacteria. The isolates were characterized by different staining techniques, and by larvicidal bioassay against *Aedes aegypti* larvae.

Result

From 282 soil samples analyzed by the acetate selection method 846 *Bacillus thuringiensis* isolates were obtained producing different types of crystal protein. Among 846 isolates only one isolate showed 100% mortality against *Aedes aegypti* larvae. On analysis of 32 soil samples by heat shock selection method, 11 *Bacillus sphaericus* and 11 *Bacillus thuringiensis* were obtained. Only one *Bacillus sphaericus* showed 25% mortality against *Aedes aegypti* larvae.

Conclusion

Two different *Bacillus* strains obtained from the soil sample of Nepal showed 100% and mild toxicity against *Aedes aegypti* larvae.

KEY WORDS

Bacillus sphaericus, Bacillus thuringiensis, Control, Nepal, Vector

INTRODUCTION

The important property of the bacteria that was discovered about 56 years ago was the mosquito larvicidal property of *Bacillus thuringiensis* (Bt). It was isolated from the dead mosquito larvae and found to produce a toxin that is toxic only to the mosquito larvae.[1,2] It appeared to be a new variety with high mosquito larvicidal activity. Later it was identified and designated as *Bacillus thuringiensis* subsp. *israelensis* (Bti) serotype H14.[3] Different strains of Bt produce a crystal protein attached to the spore or liberated in the substrate, which has specific toxicity against different insect pests. *Bacillus sphaericus* (Bs) Neide first discovered in 1965 was isolated from moribund 4th instar mosquito larvae collected near Fresno, California.[4] It produces a characteristic spherical spore located at the terminal end of the swollen sporangium. The spore contains the attached toxin. Both Bt and Bs are Gram-positive endospore-producing, saprophytic bacteria.

Since then, these two bacteria isolated from the native soil or other sources formulated in various forms and utilized as a tool to control mosquito vectors in various continents.[5-10] The efficacy of these bacteria was found to be effective in controlling the mosquito vectors.[11-14] Nepal lacks these tools to date and is dependent on chemical adulticides and/or larvicide to control adults and/or larvae of mosquitoes.

Therefore, the present study aims to isolate the novel bacteria from the soil samples of Nepal by using different techniques and testing their larvicidal capacity for larval source reduction in the water sources.

METHODS

The experiment was conducted in the Central Department of Microbiology, Kirtipur, Kathmandu, Nepal from 2022 to 2023. Soil samples of 100 grams were randomly collected from the seven provinces of Nepal in a zip lock bag labeled according to place and province, 282 soil samples were transported to the laboratory and stored at room temperature for the isolation of bacteria. The Nepal Health Research Council, Ramshahpath, Kathmandu, Nepal granted ethical approval to carry out the research (Regd. No. 79/2020).

Isolation of bacteria was carried out by two methods: (i) acetate selection method, and (ii) heat shock selection method.[15] In acetate selection method, 1 gram of soil sample was inoculated to a test tube containing nine milliliters of nutrient broth (peptone 5 g/L, HM peptone 1.5 g/L, yeast extract 1.5 g/L, NaCl 5g/L.) acetated with 0.25 M sodium acetate and incubated at 28^{0}C for overnight. After incubation, the broth was heat treated at 100^{0}C by placing the test tubes in a water bath for 5 minutes. After heat treatment, the broth was tenfold serially diluted in saline solution up to 10^{-4}, and from following 10^{-2}, 10^{-3}, and 10^{-4} dilutions, 100 µl diluted broth from each dilution was spread in a nutrient agar plate by spread plate technique. The agar plate was incubated at 28^{0}C for 48 hours. Further, the isolated colonies were subcultured in nutrient agar (NA) (Peptone 5 g/L, HM peptone 1.5 g/L. yeast extract 1.5g/L. NaCl 5 g/L, Agar 15 g/L) plate to obtain the pure culture of the bacteria.

In the heat shock selection method, 10 grams of soil sample was added to 100 ml of saline solution in a conical flask homogenized manually and placed in a shaker water bath maintained at a temperature of 80^{0}C for 30 minutes.[16] After heat treatment, the suspension was tenfold serially diluted in saline solution and from 10^{-2} and 10^{-3} dilutions, 100 µl of each dilution was spread in nutrient yeast extract salt medium (NYSM) (nutrient agar enriched with 0.5g/L yeast extract, 0.2g/L $MgCl_2$, 0.01g/L $MnCl_2$, and 0.1g/L $CaCl_2$) by spread plate technique.[16] The plates were incubated at 28^{0}C for 48 hours. The isolated colonies were subcultured in NA and preserved in NA agar slant with 60% glycerol at -20^{0}C.

Identification of the isolates was done through their cultural and microscopic characteristics. The cultural characteristics of the isolates were studied by inoculating the bacteria in NA. The color, shape, size, elevation, opacity, and consistency of the colony were observed after 48 hours of incubation. To study the microscopic characteristics pure culture of the isolates obtained in nutrient agar was stained by three different staining techniques Gram staining, spore staining, and Coomassie brilliant blue staining (CBB) (0.133% Coomassie Brilliant Blue (CBB) G250 in 50% acetic acid) staining.[17-19] Biochemical properties like catalase, oxidase, and substrate hydrolysis tests like starch, gelatin, casein, and Tween 20, hydrolysis tests were performed.

A qualitative bioassay was performed to obtain entomopathogenic bacteria by rearing larvae in laboratory conditions. The eggs of *Ae. aegypti* were obtained from the Central Department of Microbiology. The eggs collected in a filter paper were dipped into the white enamel tray of size 37 x 32 x 5 cm containing 5 liters of chlorine-free water and placed at room temperature after 48 hours, the first instar larvae were collected and placed in another tray containing fresh water and the larvae were fed with a pinch of grind dog biscuits and bakers' yeast, in 9:1 ratio until the development of the 3rd instar.[20,21]

After the development of 3rd instar larvae 25 *Ae. aegypti* larvae were placed in a cup containing 100 ml of sterile distilled water. The pure culture of the isolates was scooped by using a sterile loop and 3-4 loopfuls of the culture were added to the cup and gently dissolved by stirring with the help of a sterile glass rod and incubated at room temperature for 24 hours. After 24 hours the number of alive larvae was counted.[22] The experiment was repeated by feeding the larvae with pure and fresh cultures of *Bacillus thuringiensis* (Bt) isolates producing spherical crystal protein and *Bacillus sphaericus* (Bs) producing spherical spore with terminal swollen sporangium to observe the

bacteria causing mortality of the larvae. Quantitative bioassay as a confirmatory test for larvicidal activity, fresh cultures of larvicidal positive *Bacillus thuringiensis* and *Bacillus sphaericus* isolates were fed to the larvae as mentioned previously in four replicates incubated at room temperature along with the negative control i.e., four cups containing 25 larvae in each, with 100 ml sterile distilled water without the bacterial culture and fed. The mean mortality percentage was calculated by using the formula,

Mortality (%) = (X-Y)/X x 100

Where X = percentage survival in the untreated control and Y = percentage survival in the treated sample.[23]

Results were presented in tables, and photographs where applicable, bioassay was performed in four replicates, and the mean mortality percentage was calculated.[23]

RESULTS

By qualitative bioassay, only one *Bacillus thuringiensis* (Bt) coded as Bt-8P1A showed toxicity against *Ae. aegypti* mosquito larvae. On analysis of 32 soil samples by heat shock method to isolate *B. sphaericus* (Bs), 11 Bs isolates were obtained and only one showed weak toxicidal activity against *Ae. aegypti* larvae coded as Bs-10P5B (Table 1). The mean mortality percentage was calculated by comparing with the negative control in four replicates and the mean mortality percentage was shown by two larvicidal isolates, 100% mortality by Bt-8P1A and only 25% mortality by Bs-10P5B. In negative control cups, all the larvae (a total of 100 larvae) were alive after 24 hours without being fed. This confirms the two isolates were larvicidal bacteria.

Table 1. The number of larvicidal bacteria isolated from soil samples

Types of isolates	Isolates / soil sample	Larvicidal	Mortality %
Bacillus thuringiensis (Bt)	846/282	1	100
Bacillus sphaericus (Bs)	11/32	1	25

Total Bt isolates obtained from 282 soil samples were 846 among them 523 produced spherical-shaped crystal protein, 309 produced cap-headed or spore attached, 11 produced oval-shaped, and 2 produced bipyramid-shaped. Only one single Bs was isolated by the acetate selection method and was nontoxic to mosquito larvae. The other 10 Bs isolates did not show toxicidal activity even though the microscopic morphology of all the Bs isolates remained the same. The colony morphology of the two larvicidal bacteria was different in NA as shown in table 2. and figure 1 (A and B). Microscopic morphological characteristics of both the isolates showing larvicidal activity were Gram-positive and spore-producers, variation is seen in spore when stained by spore staining technique figure 2 (A and B).

Table 2. Colony characteristics of larvicidal bacteria on solid agar media (NA)

Isolates	Colony shape	Size	Color	Margin	Consistency	Elevation	Opacity
Bt-8P1A	Irregular	> 80 mm	Dirty white	Undulate	Dry	Flat	Opaque
Bs-10P5B	Oval / spherical	> 15 mm	Dirty white	Smooth	Mucoid	concave	translucent

In the case of Bt-8P1A, the spore location was terminal and the shape of the spore was oval (Fig. 2B). In the case of Bs-10P5B, the location of the spore was terminal inside the swollen sporangium and the shape of the spore was spherical (Fig. 2A). On staining with CBB stain a spherical crystal protein was observed in the case of Bt-8P1A (Fig. 3A) and in the case of Bs-10P5B, the spherical spore itself stained as dark blue in color due to the attachment of the crystal protein in itself (Fig. 3B). Other Bt isolates also produced spores of different shapes and were Gram-positive in addition they produced different shapes of crystal protein that was observed after staining the culture with CBB stain.

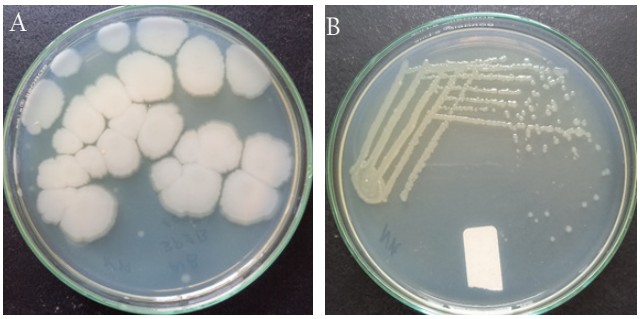

Figure 1. Colony morphology A. Bt-8P1A and B. Bs-10P5B in NA after 48 hours of incubation in agar medium.

All the Bt and Bs isolates were catalase, and oxidase positive, they all were able to hydrolyze starch, casein, Tween 20, and gelatin substrate.

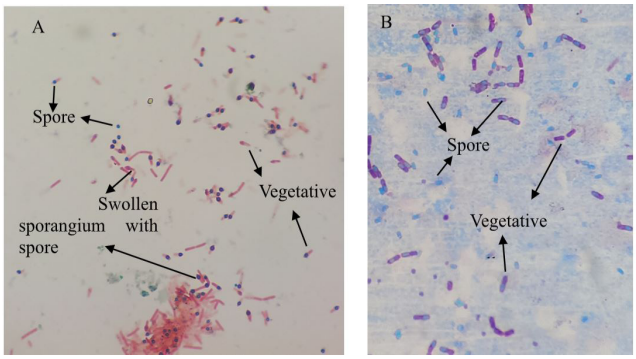

Figure 2. Microscopic characteristics of spore staining vegetative cells are stained reddish and the spherical spores are stained bluish. A. Bs-10P5B. B. Bt-8P1A.

Enrichment techniques, acetate selection, and spore selection or heat shock method were successful for the isolation of the larvicidal strains as well and the selective media used for Bs was efficient for the isolation of Bs. The media used for the isolation of Bs was NYSM agar media which encouraged the growth of Bs. In addition to Bs, this media supported the growth of eight bipyramid and three capheaded crystal protein-producing Bt bacteria.

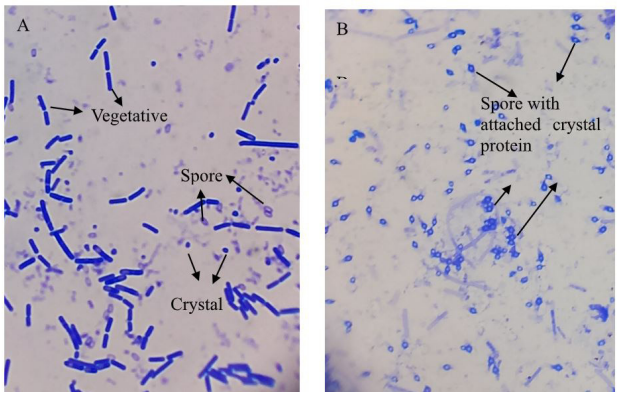

Figure 3. Microscopic observation of CBB stained crystal protein of larvicidal bacteria. A. Bt-8P1A dark blue stained rod-shaped vegetative cells, spherical-like crystal protein, and light purple empty oval spores are seen. B. Bs-10P5B blue-stained spherical ring empty spores are seen

DISCUSSION

Biological control of mosquito vectors protects people and the environment from exposure to hazardous chemicals and is an eco-friendly method. The biological control agents used are naturally occurring predatory fish *Gambusia affinis*, *Poecilia reticulata* predatory cyclopods *Mesocyclops aspericornis* larvicidal bacteria *B. thuringiensis*, *B. sphaericus* focused to control the larval stage of mosquitoes and are used in different parts of the world to control mosquitoes.[24,25] Two bacterial strains *Bacillus thuringiensis* var *israelensis* (Bti) AM 65-52 and *Bacillus sphaericus* 2362 are recommended by the World Health Organization as a biopesticide to control mosquito vectors.[13,26] In various field trials using *Bacillus thuringiensis var israelensis* (Bti) and *Bacillus sphaericus* (Bs) the success rate was higher compared with other biological control agents.[11,14,27] The present study aims to obtain novel indigenous mosquito larvicidal Bt and Bs from the soil sample of Nepal. Qualitative bioassay of 523 spherical-shaped crystal protein producing Bt only one Bt coded as Bt-8P1A showed toxicity against the *Aedes aegypti* larvae. It showed 100% mortality of *Ae. aegypti* larvae by quantitative bioassay in comparison with negative control. Among 12 Bs isolates, one Bs caused 25% mortality of *Ae. aegypti* larvae by quantitative bioassay. A lower percentage of mortality is observed in the case of Bs against *Aedes* larvae. Various literatures have shown that Bs is active against *Culex* spp and *Anopheles* spp.[25,28] The initial discovery of Bs in the past was also a weak larvicidal Bs.[28] Even though, Bs was discovered in 1965 earlier than Bt, its importance was known only after the discovery of highly toxic Bti in 1977. We found the weak larvicidal Bs isolated from the soil sample of Nepal, it may show high insecticidal activity against the *Culex* spp. The frequency of potent larvicidal Bs isolation is less, single Bs isolated from a large sample size. A greater chance of isolation of high insecticidal Bs or Bti is from the collection of dead mosquito larvae.[29,30] The sampling site is comparatively different in the present study as most of the study shows the sample was collected from a place where mosquito larvae exist or dead mosquito larvae or near a water source.[16,30,31] Further research is required to survey natural larval habitats for the isolation of novel and high insecticidal Bs. Due to unawareness and unavailability of the biological control agents, the evaluation and efficacy of the biological control agents have not yet been determined in the context of Nepal.

All the 32 soil samples analyzed for the isolation of Bs were not positive in comparison with Bt isolates, maybe the sample size used for the spread plate technique was insufficient, the organisms in the soil samples were fewer in number or the sampling site from where the sample was collected was not the appropriate niche of the organism, so the result is not in agreement.[16] Isolation of Bt from the soil sample by acetate selection method showed that every soil sample on average contains > 3 Bt organisms producing different types of crystal protein the result is consistent.[32] Acetation of nutrient broth with 0.25 M sodium acetate prevented the germination of Bt spores but encouraged the other spores to germinate during the overnight incubation. The vegetative cells were killed on exposure at 100^0C for 5 minutes still, the Bt spores were heat resistant and germinated when spread in the NA plate. The acetate selection method is found to be an excellent and most followed technique for the isolation of Bt from various samples. The heat selection method or spore selection method along with the selective media NYSM was satisfactory for the isolation of Bs from the soil samples.

The colony morphology of both the larvicidal isolates was different even though both were *Bacillus* species. However, the colony morphology of Bs and the bipyramid crystal protein-producing Bt bacteria was the same during the study. They are well distinguished by observing the spore arrangement, location, and shape of the spore of the bacteria by spore staining and observing in a light microscope or by Gram staining after 48 hours of incubation of bacterial growth. Microscopic morphology study by CBB staining of Bt and Bs revealed that the presence of spherical crystal protein in Bt liberated or expelled in the substrate and in the Bs is indicated by the dark stain around the spherical spore.

Due to insufficient facility to rear *Culex* spp we were unable to show the exact mean mortality caused by Bs against the mosquito larvae. This research paper indicates the presence of larvicidal Bt and Bs in the soil samples of Nepal and

analysis of different sources will lead to the isolation of high larvicidal Bs. A major feature present in the bacteria was tested by larvicidal bioassay under laboratory conditions against the laboratory-reared *Ae. aegypti* larvae. Further, the efficacy by field trial, molecular characterization, and the formulation of the Bt and Bs has to be carried out before the implementation of this bacteria as a biological control agent to control mosquito vectors in Nepal.

CONCLUSION

Two important mosquito larvicidal indigenous bacteria *Bacillus thuringiensis* (Bt) and *Bacillus sphaericus* (Bs) were isolated from the soil samples of Nepal. The technique used for the isolation was efficient and Bt showed 100% toxicity and Bs showed mild toxicity against *Ae. aegypti* larvae under laboratory conditions. Bt so, obtained can be used to reduce the mosquito larvae present in different water storage containers.

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