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MORPHOLOGICAL FEATURES OF MOSQUITO LARVICIDAL *BACILLUS THURINGIENSIS* ISOLATED FROM SOIL SAMPLES OF NEPAL

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ABSTRACT

Anopheles, Culex, and *Aedes* spp are the main vectors for transmitting malaria, Japanese encephalitis, lymphatics filariasis, dengue, zika, chikungunya, etc. Mosquito-borne diseases are increasing especially dengue in Nepal. Additional tools are required to decrease the disease rate. Biological control tools like *Gambusia* fish, Cyclopods, *Bacillus thuringiensis,* etc., have not been adopted and are unavailable in Nepal. The research aims to isolate mosquitocidal *Bacillus thuringiensis* a biological control tool from the soil samples of Nepal.

Bacillus thuringiensis (Bt) was isolated from the soil samples of Nepal by the acetate selection method. The larvicidal bioassay of Bt was studied against the field-collected mosquito larvae. The isolate showing larvicidal activity was coded as *Bacillus thuringiensis* -14P2A (Bt-14P2A). The growth pattern of the positive control strain *Bacillus thuringiensis* var *israelensis* (Bti-IPS-82) and the test strain were studied in different types of culture media. The microscopic morphology was studied by using different staining techniques.

Bt-14P2A and Bti -IPS-82 caused 100% mortality of field-collected larvae. Both the strains showed the same type of colony morphology in a standard culture media and uniform turbidity with sedimentation type of growth pattern in broth media. The strains were Gram-positive single rod-shaped of size 1.2X4.8µm in both negatively stained slides and Gram-stained slides. A slight difference in the endospore location was terminal in Bti-IPS-82 and subterminal in Bt-14P2A.

The newly isolated Bt-14P2A differs from Bti-IPS-82 in arrangement and endospore location but harbors the same morphological characteristic and larvicidal activity as the positive control strain.

Keywords: bacillus thuringiensis, endospore, larvicidal, Nepal, subterminal

INTRODUCTION

Climate change and an increase in vector-borne diseases (VBDs) are challenging the vector control strategies followed to date. New interventional tools should be introduced and an integrated vector management (IVM) program must be implemented to reduce VBDs in Nepal. The main strategies adopted for effective control of vector-borne diseases are disease management with early diagnosis and treatment, community awareness, and vector control (Shrestha *et al.,* 2019). Vector control practices followed in Nepal are the distribution of long-lasting insecticidal treated bed nets (LLINTs), Indoor residual spraying, elimination of breeding sources (practice only for *Aedes* spp), and surveillance of disease-transmitting vectors (Shrestha *et al.,* 2019). To achieve long-term and sustainable control of VBDs various other tools or techniques must be integrated, like environmental management (modification, manipulation of environment) (ii) Biological control agents (Fish, Bacteria, Cyclopods, etc.) (iii) chemical control (chemical insecticide) (World Health Organization, 2011), along with the conventionally used tools and techniques. More and newer intervention tools and approaches are the requirements for IVM to address the expansion of mosquito vectors.

Bacillus thuringiensis is a Gram-positive rod-shaped endospore-producing bacterium during sporulation it produces a crystal protein. The bacteria are widely distributed in various natural resources. Bt strains produce a wide variety of crystal proteins active against diverse insect pests (Palma *et al.,* 2014). Various studies on Bt strains of Nepal and their molecular characterization showed that diverse Bt strains are present in the soil samples of Nepal (Limbu *et al.,* 2020; Parajuli *et al.,* 2015; Sharma, 2017; Sijapati *et al.,* 1970). The Bt isolates from the soil sample showed toxicity against the agricultural pest *Galleria mellonella* (Limbu *et al.,* 2020). Similarly, bipyramid-shaped crystal protein-producing Bt isolates obtained from the soil samples of Nepal showed larvicidal activity against the pest *Pieris brassicae nepalensis* and *Helicoverpa armigera Hubner* (Rana *et al.,* 2002). Mosquito larvicidal Bt is used for larval source reduction in the aquatic habitat (World Health Organization, 2013). Reduction of larval

sources prevents the development of adult mosquitoes which reduces the transmission of mosquito-borne diseases. Larvicidal Bt when added to the breeding habitat of mosquitoes the larvae feed on the toxin produced by the Bt. Ingestion of toxins causes the death of larvae.

Since the discovery of *Bacillus thuringiensis* var *israelensis* (Bti) in 1976, extensive research has proven its efficacy in controlling mosquitoes (Boisvert, 2005). Larvicidal Bt has been isolated in various parts of the world formulated in different substrates and available in different forms (liquid, solid formulation) for controlling mosquito vectors (*Aedes* spp, *Anopheles* spp, *Culex* spp). For instance, Bti H-14 liquid formulation effectively reduced *Aedes* spp larval density in household containers in Denpasar, Bali (Purnama & Kardiwinata, 2021). Bti H-14, water dispersible granule, formulation was effective in the field for at least 35 days with more than 80% reduction in *Aedes* larvae (Lee & Zairi, 2006). Bti C4P1 solid formulation developed by Far –Manguinhos –Fiocruz, Rio de Janeiro, Brazil, evaluated against *Aedes aegypti* under stimulated field conditions showed 40-54 days residual activity in containers placed in a shade (Melo-Santos *et al.,* 2001). Efficacy of Bti (Bactivec) and *Bacillus sphaericus* (Griselest) to control *Anopheles gambiae* complex, *Culex quinquefasciatus,* and *Aedes aegypti* larvae under semi-field conditions in northeastern Tanzania, Bti provided 91-100% larval mortality within 24 hours whereas *Bacillus sphaericus* resulted in 98-100% larval mortality within the same timeframe (Derua *et al.,* 2022).

In Nepal application of larvicidal Bt is not yet practiced may be due to its unavailability. Even though the standard operating procedure has been developed by the Department of Health Services Nepal, to control dengue vectors by using Bti (Epidemiology and Disease Control Division, 2022). So, the research aims to isolate larvicidal Bt from the soil samples of Nepal. The main focus of the research is to isolate potent bacteria *Bacillus thuringiensis* from the soil sample as a biological control agent to control mosquito vectors as a new intervention for future implementation in vector control programs. Biological control agents like *Bacillus thuringiensis, Bacillus sphaericus,* etc. can be integrated with vector control strategies. Using biological control agents prevents the pollution caused by the chemical larvicide. Prevent bioaccumulation and biomagnifications of chemicals.

METHODS AND MATERIALS

Standard Strain *Bacillus Thuringiensis* Var *Israelensis* (Bti)

The standard reference strain *Bacillus thuringiensis* var *israelensis* (Bti)-IPS-82 was provided by the Pasteur Institute, Paris, France. The control strain was cultured in the laboratory condition in nutrient agar (NA) (Peptone 5g/L, HM peptone 1.5g/L. yeast extract 1.5g/L. NaCl 5g/L Agar 15g/L).

Bacillus Thuringiensis Isolation

Bacillus thuringiensis was isolated from the soil samples of Nepal. The soil samples were collected from seven provinces of Nepal in a ziplock bag. The sample was transported to the Central Department of Microbiology, T.U, Kirtipur, Kathmandu for isolation of Bt. The acetate selection method was performed to isolate the Bt strain (Travers *et al.,* 1987). The isolates were identified as Bt by observing Coomassie brilliant blue stained (0.133% Coomassie Brilliant Blue (CBB) G250 in 50%acetic acid) (Rampersad *et al.,* 2002) crystal protein and its shape in light microscopy from a 48 hours nutrient agar culture plate.

Growth Characteristics of Bti-IPS-82 and Bt-14P2A

The growth pattern of Bti-IPS-82 and Bt-14P2A was studied by inoculating a single colony in the following broth media, Luria Bertani broth (LB broth) (Tryptone 10g/L, yeast extract 5g/L, NaCl 10g/L.), Nutrient broth (NB broth) (Peptone 5g/L, HM peptone 1.5g/L, yeast extract 1.5g/L, NaCl 5g/L.), and in T3 broth (Tryptone 3g/L, Tryptose type I 2g/L, yeast extract 1.5g/L, Sodium Phosphate 0.05M, Manganese chloride 0.005g/L.) from a pure culture plate, and incubated for 48 hours at 28^0C in an incubator to observe the growth characteristic of the organism in broth media as well as to confirm larvicidal activity. The organism was inoculated by performing quadrant streaking to obtain an isolated colony in the nutrient agar (NA) to observe the colony morphology of the organism and also in T3 agar (Tryptone 3g/L, Tryptose type I 2g/L, yeast extract 1.5g/L, Sodium Phosphate 0.05M, Manganese chloride 0.005g/L Agar 15g/L) to confirm whether it stimulate sporulation (Bello *et al.,* 2016; Martin & Travers, 1989). Agar plates were incubated at 28^0C for 48 hours in an incubator. The sporulating property was confirmed after 48 hours by performing Coomassie Brilliant Blue staining and by spore staining from the colony present in T3 and NA agar plates as well as from their broth

culture tubes of Bti-IPS-82 and Bt-14P2A. Triplicate slides were prepared by smearing from the three isolated colonies present in the agar plate of both strains and from the broth culture and observed in a 100X objective lens of a light microscope.

Microscopic Characteristics of Bti-IPS-82 and Bt-14P2A

To observe the microscopic characteristic of Bti-IPS-82 and Bt-14P2A the organism was cultured in a basal media NA for 24 to 48 hours. From the isolated colony Gram staining (crystal violet, Gram's iodine, decolorizing agent {alcohol} and safranin) and Negative staining (an acidic dye, Nigrosin) were performed. Spore staining (Malachite green, decolorizer {water} and safranin) and CBB staining (0.133% Coomassie Brilliant Blue (CBB) G250 in 50%acetic acid) were performed from a 48 hours culture plate. The slides were prepared in triplicate from three isolated colonies.

Size Determination of Bti-IPS-82 and Bt-14P2A

The vegetative cell size was determined from the negatively stained slide and Gram stained slide prepared from a 24 hour NA culture plate. For the size determination, the ocular micrometer was calibrated with the stage micrometer in a 100X objective lens of a well magnifying light microscope Olympus Japan model CHD available in the Central Department of Microbiology. The number of ocular divisions that coincided within the vegetative cell was counted, five different mature vegetative cells were measured and the average size of the vegetative cell was calculated.

Calibration of Ocular Micrometer

One ocular division = (No. of stage micrometer division / No. of ocular division coincided with the stage division) X10µm (Centers for Disease Control and Prevention, 2015).

Larval Source

The immature (Larva, Pupa) was collected from the discarded tires using a strainer and placed in a plastic cup with the help of a dropper and transferred to a transparent ziplock bag containing clean water and transported to the laboratory. In the laboratory, the immatures were placed in a white enamel tray containing fresh water and fed with a pinch of dog biscuit and baker's yeast (nine: one) mixture made to a fine powder. The pupae were collected from the tray and placed in a cup until the emergence of adults and identified.

Larvicidal Activity

The isolated Bt strains producing spherical crystal protein were screened for larvicidal activity by inoculating 3 loopfuls of 48 hours cultures of Bt strain in a cup containing 10 mosquito larvae (third and fourth instar larvae), and 100 millilitre (ml) of distilled water. The mosquito larvae were not provided with larval food during the bioassay (Bello *et al.,* 2016; Lakxmy *et al.,* 2011). The cup was incubated at room temperature for 24 hours. Mortality of larvae was observed after 24 hours. The Bt strain was further subcultured in NA for preservation and in three different tubes containing NB (Peptone 5g/L, HM peptone 1.5g/L. yeast extract 1.5g/L. NaCl 5g/L.), LB, and in T3 and incubated for 48 hours in an incubator. After 48 hours the broth was screened for larvicidal activity by inoculating 1000 microliter of the broth in a cup containing 10 larvae, and 100 ml distilled water. Similarly, the negative control cup containing only 10 larvae with 100 ml distilled water, and the positive control cup containing 10 larvae, 100 ml distilled water, and 1000 microliter of the standard Bti-IPS-82 cultured in NB broth was added and incubated along with the test cups for further confirmation of its larvicidal activity.

RESULTS AND DISCUSSION

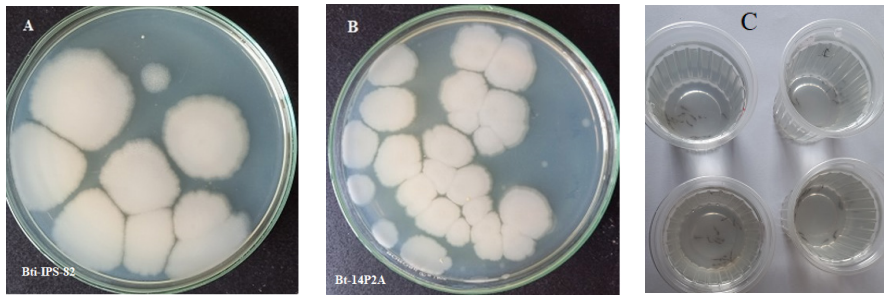
On analysis of 300 soil samples collected from seven provinces of Nepal, 1000 Bt isolates were obtained. The Bt was distinguished from the other *Bacillus* species by observing the presence of crystal protein stained by CBB stain (Bravo *et al.,* 1998; Rampersad *et al.,* 2002). Generally, after growth for 48 hours in NA agar, all the *Bacillus* start to sporulate, on staining the isolated colony from the culture plate by CBB stain. In the case of the Bt strain, three different types of morphological structure can be observed in a slide, the vegetative cell, the spore, and crystal protein. In the case of other *Bacillus* spp, only two morphological structures can be observed the vegetative cell and, the spores. The presence of the extra structure of different shapes confirms it as a Bt strain the structure is the protein and it is stained by the CBB stain mainly used for staining the protein structure (Rampersad *et al.,* 2002). From the 1000 isolates, the majority of the isolates produced spherical shape crystal protein 55%, followed by cap-headed or spore attached 44%, and only two isolates produced bipyramid crystal protein 0.2%. The result is consistence with (El-Kersh *et al.,* 2016) On screening for larvicidal activity against the field-collected mosquito larvae, only one Bt coded as Bt-14P2A showed larvicidal activity. Various study has shown only the presence of a few isolates of larvicidal Bt from

soil samples for instance (Lobo *et al.,* 2018) obtained 3 larvicidal Bt from 300 Bt isolates of soil samples. So, the larvicidal assay distinguishes the mosquito larvicidal Bt from the other Bt strains as the crystal proteins are specific to a target pest (Bravo *et al.,* 1998). As the composition of the crystal protein varies from one species to another so, this characteristic is also an important characteristic to distinguish among the Bt strain (Xu *et al.,* 2014). The larvicidal Bt strain was isolated from the soil sample of Province 2 of Nepal. The addition of 1000 microliters culture broth of LB, NB, and T3 to each cup containing 10 larvae showed 100% mortality of larvae within 24 hours (Figure 1C). So, all three types of broth substrate encourage the growth of the larvicidal Bt, and production of toxin. This confirms the isolates Bt strains as a mosquito larvicidal Bt. The larvicidal activity was not only shown by the organism after growth in the solid agar media but also in the liquid broth containing the crystal protein toxin to mosquito larvae. This confirms the isolate as a mosquito larvicide. The pupae after emergence to adulthood were identified as *Aedes aegypti*, *Culex*, and *Aedes albopictus.* Thus Bt-14P2A is effective in controlling *Culex* and *Aedes* spp.

Bacillus thuringiensis var *israelensis* as a positive control strain and Bt-14P2A as a test strain, both the strains showed the same type of colony morphology after 48 hours of incubation at 28^0C in a solid agar (NA) media, the standard media to study the colony characteristic of bacteria (Table 1). A similar type of growth pattern of the Bt-14P2A and positive control in the culture media indicates the presence of toxic crystal protein required for larvicidal activity. Observation of similar colony morphology would also help to isolate numerous larvicidal Bt from different sample sources in the future (Figure 1A, 1B). In the LB, NA, and in T3 broth uniform turbidity with sedimentation type of growth pattern was observed in both strains. The growth pattern in the broth media will help in the formulation of Bt. Turbid growth indicates a uniform distribution of the organism and the crystal protein in the broth or liquid formulation which will get easily dispersed while used as a bio-pesticidal agent or mosquito larvicidal agent in aquatic habitat. After 48 hours of incubation in the T3, NB, and LB broths visible turbidity was observed. The triplicate slides observation proved that T3 media was an excellent spore stimulating media as the 3 slides contained spore and the crystal only (Figure 2) no vegetative cells were observed so the result states within 48 hours all the vegetative cells sporulated than in NA.

Figure 1

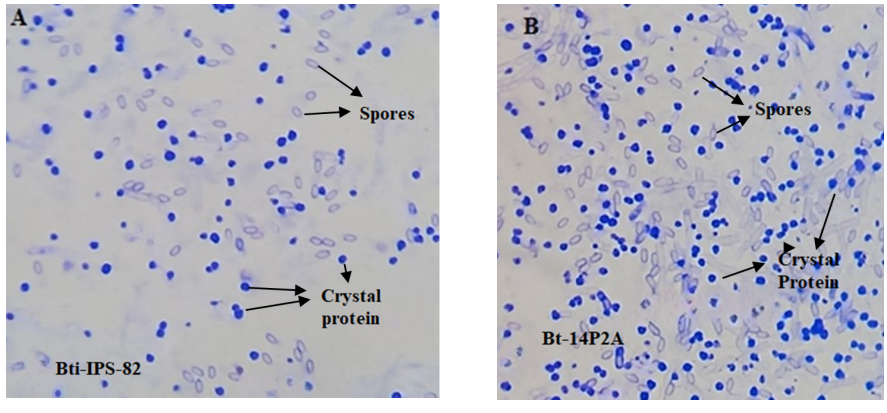
A and B Isolated colonies in nutrient agar plate after 48 hours incubation A. Bti-IPS-82 B. Bt-14P2A. C. Larvicidal bioassay Bti-IPS-82 and Bt-14P2A in a plastic Cup.



In the case of larvicidal or entomopathogenic Bt faster sporulation results in the accumulation of crystal protein in the substrate therefore the spore and crystal protein mixture increase the efficacy of the formulation. The results conclude T3 composition should be used for the formulation of the bio-pesticides. However, in the case of strains isolated in the NA plate, all three morphological structures (vegetative cell, spore, sporulating cell, and crystal protein) can be observed in a slide preparation even after 48 hours of incubation.

Figure 2

CBB stain crystal protein and the spore from T3 agar plate after 48 hours. Crystal proteins are stained as dark blue irregular or spherical like in appearance and the spores are elliptical empty slightly blue in color.



The morphological characteristic colony, and microscopic morphological characteristics were studied by following the guidelines (WHO Environmental Health Criteria 217, 1999). Microscopic morphological observation of a Gram stain slide showed the organisms are Gram-positive single rod-shaped no spore and sporulating stage was observed in a 24 hours NA culture plate of both the strains.

Table 1

Growth Characteristics on Solid Agar (NA) and in LB Broth

Isolates	Shape of the colony	Size	Color	Margin	Consistency	Elevation	Opacity	LB
Bti-IPS-82	Irregular	>80mm	Dirty white	Undulate	Dry	Flat	Opaque	Sediment and turbid
Bt-14P2A	Irregular	>80mm	Dirty white	Undulate	Dry	Flat	Opaque	Sediment and turbid

The average size was found to be 1.2X4.8µm in both strains. The same measurement was obtained in a negatively stained slide as well as in Gram stained slide of both strains.

On observation of a Gram stain slide or a negative stain slide different sizes of vegetative cells were present in the slide prepared (Figure 3) from a 24 hours culture plate this may be due to the division of the cell. Rod shaped bacterial cells divide or multiply by transverse binary fission so the cells that have recently divided are in smaller size than the cells that have attained maturity. The matured cell size was measured and tabulated. Thus, the Bt-14P2A size is the same as the control Bti-IPS-82. One of the important characteristics of Bt is they all produce endospores. The spore inside the vegetative cell is an endospore. Spore staining of both strains showed a slight difference in the location and arrangement of the spore inside the vegetative cell. In the case of Bti-IPS-82, the endospore location is terminal the spore is attached to the lateral side wall of the vegetative cell, and a short constriction is observed between the fore spore cell and the mother cell (Figure 3B and 3C).

Table 2

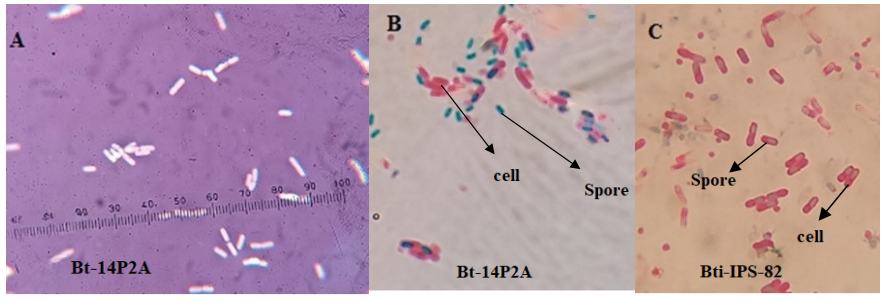
Size of the Larvicidal Bt Cells After 24 Hours by Gram Stain and Negative Stain

Isolates	Negative stain		Gram stain		Spore stain	
	Width	Length	Width	Length	Shape of spore	Location of spore
Bti-IPS-82	1.2µm	4.8µm	1.2µm	4.8µm	Elliptical	Terminal
Bt-14P2A	1.2µm	4.8µm	1.2µm	4.8µm	Elliptical	Terminal

Whereas in the case of Bt-14P2A, the spore location is sub-terminal and it is centralized in the vegetative cell (Figure 3B). However, the shape of the spore is the same elliptical (Table 2) in both strains.

Figure 3

A. Negative staining of Bt-4P2A transparent vegetative cells is seen in a bluish background. B. Spore staining of Bt-14P2A, green ellipticals are liberated spores and red stained vegetative cells. C. Spore staining of Bti-IPS-82, green elliptical spores inside the vegetative cell, and red stained vegetative cells.



Limitations of the study were a well-developed mosquito rearing facility was not available to rear mosquitoes of different genera to produce a large number of uniform larvae for the larvicidal bioassay. For larval sources in Kathmandu, has to wait for the summer season to collect larvae from the field. To study the morphology of the crystal protein by SEM (Scanning Electron Microscope) is not available in Nepal and the sample has to be sent to other Laboratories. The isolated Bt gene has to be further analyzed.

CONCLUSION

The morphological characteristics of Bt-14P2A were similar to the standard control strain Bti-IPS-82, like the colony morphology in

the NA agar plate, the size of the cell, the shape of the crystal protein, and the elliptical spore. The Bt-14P2A strain is as effective as Bti-IPS-82 in killing the mosquito larvae but differs in arrangement and location of spore. Bt-14P2A will be effective in controlling mosquito-borne diseases as it has shown the mortality of dengue vectors and *Culex* mosquito larvae during the study. The isolate Bt-14P2A produces the toxic crystal protein in all three types of broth culture substrate as well as in the agar plate. The method followed for isolation of Bt from soil samples and identification by CBB was a reproducible and reliable method.

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