ISSN (Online): 2091-2609
DOI Prefix: 10.3126/ijasbt

International Journal of Applied Sciences and Biotechnology (IJASBT)

Short Communication

Screening and Confirmation of *Vip3* gene presence in *Bacillus thuringiensis* strains native to Nepal

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Article Information

Received: 16 February 2022
Revised version received: 25 March 2022
Accepted: 26 March 2022
Published: 29 March 2022

Cite this article as:
S. Dahal et al. (2022) Int. J. Appl. Sci. Biotechnol. Vol 10(1): 71-74. DOI: 10.3126/ijasbt.v10i1.44162

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Keywords: *Vip3*; PCR; Insecticidal protein

Abstract

An insecticidal protein named Vegetative Insecticidal Protein (Vip) produced by *Bacillus thuringiensis* during its vegetative growth is gaining its popularity as second-generation insecticidal protein because of its lethal effect on insects of class Coleoptera, Lepidoptera, Diptera. The research was designed for the confirmation of *Vip3* gene presence in soil samples collected from different geographical region of Nepal. For the purpose, 36 soil samples from different places of Nepal were collected. Primers were used for PCR amplification of DNA templates extracted and product size was observed around 1621bp.Out of total sample analyzed, 10% of sample showed the presence of *Vip3* gene.

Introduction

Pest control in agriculture is mainly achieved using chemical insecticides. However, continuous use of these chemical pesticides in field has led to several problems, including environmental pollution and increase in human health effects, such as cancer and several immune system disorders (Bravo *et al*., 2011).

The most successful insect pathogen used for insect control is the bacterium *Bacillus thuringiensis* (Bt), which presently is about 2% of the total insecticidal market(Raymond *et al*., 2010) and best known insecticidal proteins are the Cry proteins from *Bacillus thuringiensis* accumulated in the parasporal crystal at the time of sporulation (Chakroun *et al*., 2016). Despite of wide use and success, resistance against Cry protein was observed around the globe *B. thurengiensis* also produces another form of insecticidal protein called Vegetative Insecticidal Proteins (Vip),during

its vegetative development period which demonstrate insecticidal activity against abroad spectrum of insects (El-Gaied *et al.*, 2020; Estruch *et al.*, 1996).

Among identified *Vip* genes, *vip3* genes are around 2.4kb in length, and they are normally carried on large plasmids although in some cases, they have been proposed to be located in the bacterial chromosome. Genes coding for Vip3 proteins are commonly found among *B.thuringiensis* strains, and hence, some studies have even found them in 50% and up to 87% of the strains tested (Chakroun *et al*., 2016).

Number of amino acids in any particular Vip3 proteins ~787, and the protein has an average molecular mass of ~89 kDa. The N terminus of Vip3 protein is highly conserved, while the C-terminal region is highly variable ; thus, the C-terminal region was proposed to be involved in target specificity (Chakroun *et al*., 2016; Hernández-Rodríguez *et al*., 2009; Mesrati *et al*., 2005; Yu *et al*., 2011). Estruch *et al*., (1996) proposed mode of action of theVip3 proteins so on after its discovery in 1996. It was suggested that Vip3 proteins would exert their toxicity via a process different from that of the Cry proteins, based on the lack of structural homology of these two types of proteins. Despite being so different, both types of toxins exert their toxic action through apparently the same sequence of events: activation by midgut proteases, crossing the peritrophic membrane, binding to specific proteins in the of the epithelial midgut cells, and pore formation (Chakroun *et al*., 2016; Lee *et al*., 2003).

This research aims to determine the prevalence of *Vip3* gene in the *Bacillus thuringiensis* strains native to soil of Nepal. It will also assist researchers for further work and to identify Vip3 protein as potential candidate for biopesticide against moths affecting crops of Nepal.

Materials and Methods

Sample collection and isolation

Samples were collected from different geographical regions of Nepal including high altitude of Himalayas, low land Terai and Hilly region.

Collected samples, each approximately 5gm were taken from at least 5 cm below ground level by scrapping off with sterile spatula were packed in air tight bag with proper labeling and brought to NAST lab and kept in freezer at 4ºC and processed. For isolation of *B. thuringiensis*, 1gm of soil from each sample were weighted and mixed in 10ml of autoclaved distilled water in a test tube. Test tubes were then vortexed vigorously for 3 mins for proper mixing of soil and serial dilution of each sample (upto10^{-4}) were done. All test tubes were then kept at 60^0C for an hour to eliminate vegetative and non-spore forming cells. After heat treatment 0.1 ml of aliquots of serial dilution were plated in NA plate prepared earlier using spread plate technique. Plates were incubated at 30ºC for overnight for cell growth (Rabha *et al*., 2017). Spore forming, potential bacterial isolates exhibiting *Bt* like phenotype i.e. flat, matte like colour, uneven border were selected and cultured in T3 agar media (Per liter: 3g tryptone, 2g tryptose, 1.5g yeast extract, 0.05M sodium phosphate [pH 6.8], and 0.005g MnCl) and incubated at 37ºC for 5 days for the confirmation of crystal production. Production of crystals was confirmed by Amido black staining (Hassan *et al*., 2021; Russell & Al, 1987; Shishir *et al*., 2012).

DNA Extraction

Rapid boiling method was performed. Overnight culture of *B. thuringiensis* in LB broth was centrifuged at 10,000 rpm for 2 mins and pellet were resuspended in 200 µl of autoclaved distilled water and then tube were transferred in boiling water for 10 mins and allowed to cool. Tubes were centrifuged at 10,000 rpm for 2 mins and supernatant were transferred to fresh sterile eppendorf tube and stored at 4ºC for further analysis (Diana *et al*., 2012; H. Abdelhai, 2016).

Gene Identification by Polymerase Chain Reaction (PCR)

PCR reaction was carried out in 20 µl reaction volumes containing 10 µl of 2X master mix (Thermo Scientific), 0.5µl of Taq polymerase (Promega) of 5 unit/ml, 1µl of 10 pM of reverse and forward primer,0.4 µl of $Mgcl_2$ and 5.1 µl of sterile water and 2µl of template.

Amplification was carried out in PCR thermocycler (Applied Biosystem) with program set for 35 cycles of denaturation at 95 ºC for 1 min, annealing at 51ºC for 1 min and extension at 72ºC for 1.5 mins with an extra step of extension at 72ºC for 10 mins. After PCR amplification, electrophoresis of PCR products was done at 1.5% agarose gel using DNA ladder of 1 Kb (Thermo Scientific) (Fig. 3).

Primer for reaction was used as described by Hernández-Rodríguez *et al*. (2009) sequence of which are listed below and was synthesized by Macrogen.

vip3- forward primer:
5′-TGCCACTGGTATCAARGA-3′

vip3- reverse primer:
5′-TCCTCCTGTATGATCTACATATGCATTYTTRTTRTT-3′

Result and Discussion

In our experiment, 30 crystal positive samples were identified among which 22 shows spherical crystal whereas 7 showed bipyramidal crystal and 1 showed mixed crystal i.e. 73.33% of crystals were spherical in shape, 23.33% were bipyramidal and rest 3.33 % was mixed (Mixture of bipyramidal and spherical) (Fig. 1 and 2).

Fig 1: Spore observed under microscope

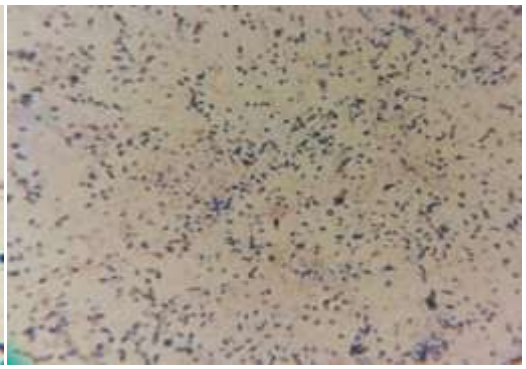

Fig 2: Crystal observed under Microscope

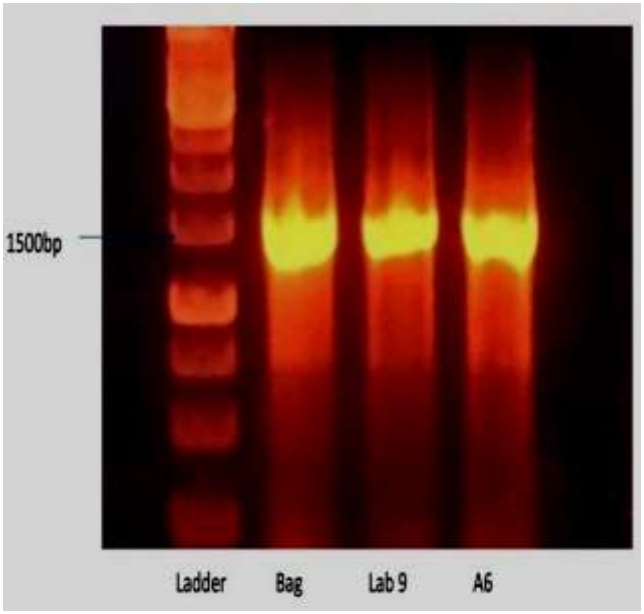

Fig 3: Agarose gel electrophoresis of PCR product**.**

From the above experiment result, prevalence of *vip 3* gene found to be 10% as we observed band at 1621bp. Among *30 Bacillus thuringiensis* samples only 3 was found to be *Vip 3* gene positive (Fig. 3). All 3 samples having *Vip 3* positive gene were obtained from mild temperate zone i.e. from altitude of 2100m-2800m. Study of Palma *et al*., (2013), abundance of *Vip3* gene was found to be 14.5% on the Spanish main land whereas 2 % in Canary island which support and justify the result of study (Palma *et al*., 2013). However in some research abundance is as high as 67.4% (Yu *et al*., 2011).

Conclusively, more research on topic needs to be done for study of temperature and other geographical parameters effects on *Vip3* prevalence and further research needs to be carried out for determination of Vegetative Insecticidal Protein as potential candidate for biopesticides.

Author's Contribution

All authors contributed equally in the all stages of research and manuscript preparation. Final form of manuscript was approved by all authors.

Conflict of Interest

The authors declare that there is no conflict of interest with present publication.

Acknowledgment

Authors express sincere gratitude and heartiest honor to Nepal Academy of Science and Technology, Khumaltar, Lalitpur.

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