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Isolation of *Bacillus thuringiensis* from Soil and their Impact on Mosquito Larvae
Aislamiento de *bacillus thuringiensis* del suelo y su impacto en las larvas de mosquito

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ABSTRACT

Mosquitoes are vectors of various disease-causing agents and are responsible for the transmission of pathogens causing more life threatening and debilitating human diseases than any other organism. Over one million people worldwide die from mosquito borne diseases which include Malaria, Chikungunya, Yellow fever and Dengue fever yearly within disease- endemic countries. Continuous use of chemical-based insecticides has resulted in the development of resistance in mosquito and cause detrimental effects on non-target organisms and thus causes human health problems. *Bacillus thuringiensis* is an important insect pathogen is highly toxic to mosquito larvae and related dipterans. *Bacillus thuringiensis* during the sporulation phase produces proteinaceous parasporal crystals called as delta-endotoxin. This crystal protein is active in alkaline condition of midgut of insects and gets solubilized and when activated by intrinsic protease results in an active toxin that selectively binds specific receptor in the cell membrane, leading to pore formation and consequent insect larvae death. Thus, the present study reveals *Bacillus thuringiensis* to be highly active on mosquito pests and less likely to cause resistance and no effect to non-target animals and is thus considered beneficial to humans in the eradication of Mosquitoes.

Keywords: Mosquitoes, Transmission, Malaria, Chemical based Insecticides and *Bacillus thuringiensis*

RESUMEN

Los mosquitos son portadores de diversos agentes patógenos y responsables de la transmisión de patógenos que causan más enfermedades mortales y debilitantes que cualquier otro organismo. Más de un millón de personas mueren anualmente en todo el mundo por enfermedades transmitidas por mosquitos, como la malaria, la chikunguña, la fiebre amarilla y el dengue, en países endémicos. El uso continuado de insecticidas químicos ha provocado el desarrollo de resistencias en los mosquitos y tiene efectos perjudiciales en organismos no diana, lo que causa problemas de salud humana. El *Bacillus thuringiensis* es un importante patógeno de insectos altamente tóxico para las larvas de mosquitos y dípteros afines. Durante la fase de esporulación, el *Bacillus thuringiensis* produce cristales proteínicos parasporales llamados delta endotoxinas. Esta proteína cristalina es activa en las condiciones alcalinas del intestino medio de los insectos, se solubiliza y, cuando es activada por la proteasa intrínseca, da lugar a

una toxina activa que se une selectivamente a un receptor específico de la membrana celular, provocando la formación de poros y la consiguiente muerte de las larvas de los insectos. Así pues, el presente estudio revela que el *Bacillus thuringiensis* es muy eficaz contra las plagas de mosquitos y tiene menos probabilidades de causar resistencia y no afectar a los animales no diana, por lo que se considera beneficioso para los seres humanos en la erradicación de los mosquitos.

Palabras clave: Mosquitos, transmisión, malaria, insecticidas químicos y *Bacillus thuringiensis*.

INTRODUCTION

Mosquitoes comprise of a group of about 3,500 species of small insects that are flies. Common insecticides applied for mosquito's elimination has given rise to problems for human and environment. Chemical insecticides provide benefit in food production, human health and have proven very effective at increasing agriculture and forestry productivities. However, uncontrolled use of chemical insecticides has resulted in irreparable damage to environment. Consequently, the need for alternative control measures arise which leaves biological control as a viable alternative to chemical control. Microbial insecticides are especially valuable because their toxicity to non-target animals and humans is extremely low and a crucial part of integrated pest management. Compared to other commonly used insecticides, they are safe for both the pesticide user and consumers of treated crops. The most useful methods for controlling these diseases based on vector control are mainly accomplished by using synthetic insecticides and use of entomopathogenic bacteria as biolarvicides can be a favourable alternative for insect control.

Bacillus thuringiensis

Bacillus thuringiensis (Bt) is a well-known and widely studied bacterium which is known for its use in pest management. Today it is the most successful commercial xenobiotic with its worldwide application when compared to the chemical pesticides. *Bacillus thuringiensis* has been used as a successful biological insecticide for more than 40 years and is a uniquely specific, safe and effective tool for the control of a wide variety of insect pests. *Bacillus thuringiensis* is an important insect pathogen which is highly toxic to mosquito larvae and related dipterans. *Bacillus thuringiensis* is selectively active on pests and less likely to cause resistance hence it is considered beneficial to humans, animals and plants and also as a suitable replacement to chemical pesticides in many countries.

Bacillus thuringiensis δ-endotoxins

Two types of δ -endotoxin are produced by Bt strains. They are Cry and Cyt proteins. Each insecticidal crystal protein is the product of a single gene. The genes synthesize these endotoxins which are often located on large, transmissible plasmids. Cry and Cyt proteins differ structurally. The most important feature of these proteins is their pathogenicity to insects and each crystal protein has its distinct host range.

Cry proteins

Cry proteins are the predominant type. The crystal proteins are encoded by cry genes. The accumulation of Cry protein in a mother cell can make up 20-30% of the dry weight of the sporulated cells. Each crystal protein has its own insecticidal spectrum. Therefore, Cry proteins have been classified on the basis of their host specificity and their amino acid compositions. The crystal proteins have different forms such as bipyramidal (Cry1), cuboidal (Cry2), flat rectangular (Cry3A), irregular (Cry3B), spherical (Cry4A and Cry4B), and rhomboidal (Cry11A). Cry1, Cry2, and Cry9 proteins show strongest toxicity to Lepidopterans. Proteins belonging to the class Cry4 and Cry11 are specifically toxic to Dipterans. Cry3, Cry7, Cry8, Cry14, Cry18, Cry34, and Cry35 proteins show insecticidal activity against Coleopterans. Some Cry proteins on the other hand display toxicity to more than one insect order. For example, Cry1I is both active against Lepidopterans and Coleopterans, whereas Cry1B shows toxicity against Lepidoptera, Coleoptera and Diptera. The molecular weight of the cry protein ranges from low molecular mass (35-66 kDa), medium molecular mass (66-80 kDa) and high molecular mass (80-116kDa.) respectively.

Action mechanism of endotoxins

Crystals are formed as protoxins by *Bacillus thuringiensis*. To become active; a susceptible insect must ingest them. After being ingested, the crystals are solubilized in the alkaline environment in the insect midgut (pH>10). After solubilization, midgut proteases convert the protoxins into active toxins. The active toxin binds to specific receptors on the membranes of epithelial midgut cells; this interaction provides the insertion of the toxin into the lipid bilayer and formation of pores (0.5 to 1 nm). As a result, pore formation leads to gut paralysis. Finally, insect larvae stop feeding and die from lethal septicaemia.

Phenotypic characterization

Phenotypic characterization methods include description of crystal morphology, biochemical reactions, bioassay and SDS-PAGE. The characterization of *Bacillus thuringiensis* is mainly based on the presence of Cry proteins that are detected by phase-contrast microscopy.

Genotypic characterization

16S rRNA sequencing was carried out to confirm the organism. The ribosomal operon is useful to trace genetic relationships and to identify strains rapidly.

MATERIALS AND METHODS

Collection of soil

Two soil samples were collected from different localities in Chennai.

Isolation of *bacillus thuringiensis* from soil samples by acetate selection method

10 ml of LB broth was prepared and buffered with 0.25M sodium acetate and autoclaved for 45 minutes. 1g of soil sample was inoculated in the broth. The tubes were kept in shaker for 24 hours. 1ml of the broth was transferred to the fresh tube and heated for 80^{o}C for 10 minutes. Then the broth was serially diluted 10^{-1} to 10^{-6} and

plated on the nutrient agar plate. After 24 hours of incubation, the growth was observed and the colonies similar to *Bacillus thuringiensis* were selected for Gram staining, Spore staining and Crystal staining.

Identification of bacillus thuringiensis by morphological and biochemical tests

Gram staining, spore staining, crystal staining, Indole test, Methyl red test, Voges Proskauer test, Starch Hydrolysis, Citric acid utilization test, Triple sugar iron test, Catalase test, Oxidase test, Gelatin test, Carbohydrate fermentation test were determined using standard protocols.

Antibiotic susceptibility test

Muller Hinton Agar was prepared and was autoclaved for 45 minutes after which it was poured into sterilised petriplates and was allowed to solidify. The bacterial inoculum suspension was streaked over the surface of the media using sterile cotton swab. Wells were made using cork borer. 20µl of penicillin, streptomycin, ampicillin, amoxicillin, gentamycin was poured in the well. Plates were incubated in incubator for 48 hours at 37^{o}C.

Isolation of plasmid DNA

A loopful of the bacterial culture was inoculated in 100ml of LB broth and was incubated for 24 hours in a shaker. 1.5ml of overnight bacterial culture was taken in a fresh eppendorf tube. It was centrifuged at 8000 rpm for 10 minutes. The supernatant was discarded and pellet was collected. 0.2ml of Alkaline lysis buffer – I was added to the pellet and was vortexed. It was again centrifuged at 8000 rpm for 5 minutes. The supernatant was discarded and 0.3ml of alkaline lysis buffer – II was added and was gently vortexed. The tubes were kept at room temperature for 5 minutes. Without any discarding 0.3ml of alkaline lysis buffer – III was added. The tubes were kept on ice for 10 minutes. It was centrifuged at 8000 rpm for 5 minutes. The supernatant was collected and equal amount of ice-cold ethanol was added and was kept at room temperature and centrifuged at 8000 rpm for 2 minutes. The supernatant was discarded and the pellet was air dried. 0.02ml of 1X TE buffer was added to dissolve the DNA. 0.01ml of gel loading dye was used and the presence of DNA was checked on 1% agarose gel by running on electrophoresis unit at 100MV for 30 minutes and viewed under UV transilluminator.

Protein extraction

A loopful of the bacterial culture was inoculated in 100ml of Nutrient sporulating medium and was incubated for 4 days in shaker. 1ml of bacterial culture was transferred to sterilized eppendorf tube containing 1M ice cold NaOH. It was re-suspended and the pellet was centrifuged for 5 minutes at 13,000 rpm and the supernatant was discarded. The pellet was re-suspended into 140µl of 1% SDS buffer and 0.01% β-mercaptoethanol and was kept for incubation at room temperature for 10 minutes. The solution was vortexed well. After incubation, the samples were centrifuged at 13,000 rpm for 10 minutes. The supernatant was discarded and 140µl of 20% TCA was added and was incubated for 10 minutes in ice. After incubation, the samples were centrifuged at 10,000 rpm for 15 minutes. The supernatant was discarded and equal amount of acetone was added to the pellet and mixed well. It was centrifuged at 12,000 rpm for 5 minutes. The pellet was dissolved in 1ml of Phosphate Buffer solution. The protein was estimated by Lowry's method.

16S rRNA sequencing

Genomic DNA isolation was carried out using CTAB for lysis followed by phenol chloroform method of DNA extraction. The genomic DNA was then used to amplify the 16SrRNA region with specific primers using taq DNA polymerase (Genei). The amplified products were then checked on 1% agarose gel for further confirmation. The amplified products were then purified using PEG purification protocol. The purified product was further taken for sequencing using ABI 3730XL with BDT ver 3.1 chemistry.

Activity of *bacillus thuringiensis* against mosquito larvae

The water was poured in the well with mosquito larvae and the cry protein was dissolved with water with various concentrations and poured into the well containing mosquito larvae.

RESULT AND DISCUSSION

Sample collection

Soil samples were collected from the different places like Thiruvottiyur, Aynavaram. The soil samples were collected from 5cm under the surface and stored in the sterile plastic bags (Figure 1). White spreaded with irregular margins colonies were isolated (Figure 2).

Figure 1: Soil sample in Sterile Plastic Bags

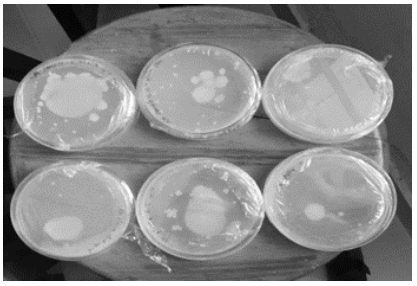

Figure 2: Colonies in Nutrient Agar

Staining

Gram staining was done to identify the Gram character of an organism. Gram positive rods were identified (Figure 3). Spores are observed green in colour and the vegetative cells are observed in red colour in Spore Staining (Figure 4). Cells, spores and crystals were observed in 100X magnification in Crystal Staining (Figure 5).

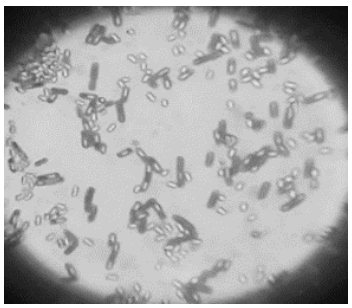

Figure 3: Gram Staining

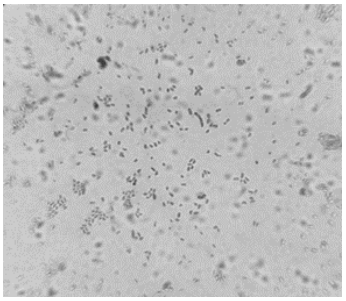

Figure 4: Spore Staining

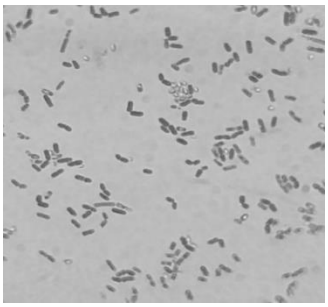

Figure 5: Crystal Staining

Biochemical tests

The following Biochemical tests were performed in the microorganism (*Bacillus thuringiensis*) isolated from the two soil samples (Sample I: Thiruvottriyur and Sample II Ayanavaram) (Table 1).

Table 1: Biochemical Tests

TESTS	Sample - 1	Sample – 2
INDOLE	Negative	Negative
METHYL RED TEST	Negative	Positive
VOGES PROSKAUER TEST	Positive	Positive
STARCH HYDROLYSIS	Positive	Positive
CITRIC ACID UTILIZATION TEST	Positive	Positive
TRIPLE SUGAR IRON TEST	Red slant, Yellow butt	Red slant, Yellow butt
GELATIN	Positive	Positive
CATALASE	Positive	Positive
OXIDASE	Negative	Negative

Carbohydrate fermentation test

Colour changes indicates positive for fermentation of carbohydrate maltose in the media. Absence of colour changes indicates negative for fermentation of carbohydrate lactose in the media and colour changes indicates positive for fermentation of carbohydrate maltose in the media.

Antibiotic sensitivity test

The Antibiotic Sensitivity Test revealed Penicillin and Ampicillin to be resistant to Organism I and II (Table 2 and Figure 6).

Table 2: Antibiotic Sensitivity Test

ANTIBIOTICS	SAMPLE - 1	SAMPLE - 2
Penicillin	Resistant	Resistant
Ampicillin	Resistant	Resistant
Amoxicillin	Sensitive	Resistant
Streptomycin	Sensitive	Sensitive
Gentamycin	Sensitive	Sensitive

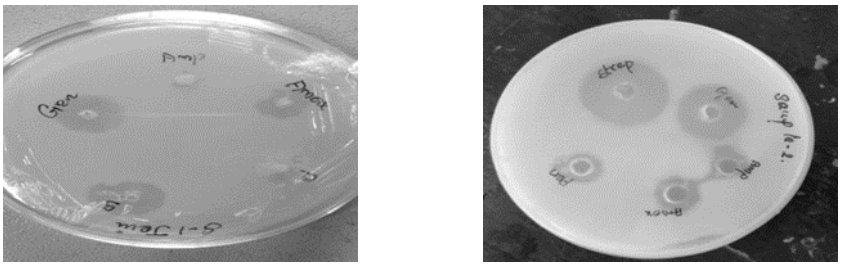

Figure 6: Antibiotic Sensitivity Test

Isolation of plasmid DNA

Plasmid was isolated and orange bands were observed under UV transilluminator. The orange band indicates the presence of plasmid DNA (Figure 7).

Estimation of protein by lowry's method

The blue colour observed indicates the presence of the protein (Figure 8).

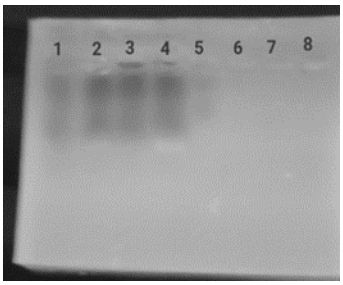

Figure 7: Plasmid DNA Estimation of Protein

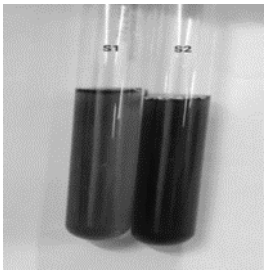

Figure 8: Estimation of Protein By Lowry Method

16S rRNA sequencing

BLAST result showed that the organism was similar to *Bacillus thuringiensis* with 99.27% similarity and E value 0.0 (Figure 9).

Bioassay

Isolated *Bacillus thuringiensis* (Samples 1 and 2) were investigated for their potential activity against mosquito larvae. The mortality caused to the mosquito larvae was 100% after the duration time of 2 hours (Table 3).

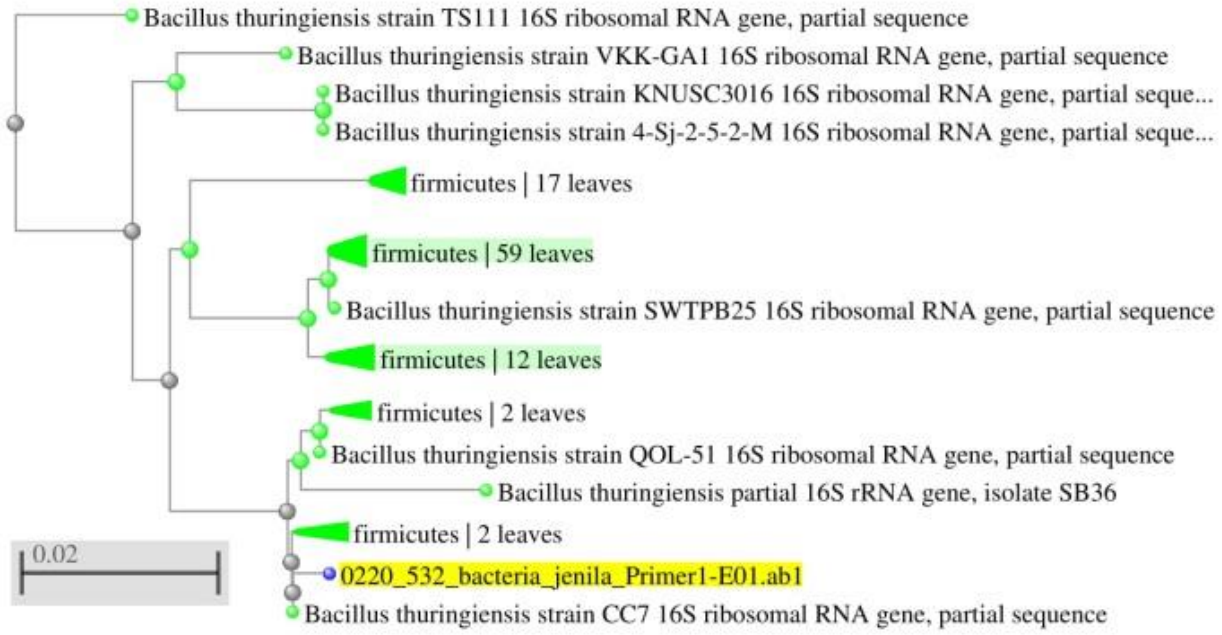

Figure 9: Phylogenetic Tree

Table 3: Bioassay

Dilution factor	1 hour				2 hours			
	Sample – 1		Sample – 2		Sample - 1		Sample – 2	
	Live larvae	Death larvae	Live larvae	Death larvae	Live larvae	Death larvae	Live larvae	Death larvae
10^0	0	3	3	3	0	3	0	3
10^2	2	1	1	2	0	3	0	3
10^4	3	0	3	0	0	3	0	3
10^6	2	1	3	0	0	3	0	3
10^8	3	0	3	0	0	3	0	3

Thus, the present study research findings clearly indicate that *Bacillus thuringiensis* is highly active on mosquito pests and less likely cause resistance and no effect to non-target animals and is thus considered beneficial and safe to humans in Mosquito Control.

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